

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Sinan Tas and Oktay Avcı
Application No.:	10/682584
Filed:	October 9, 2003
For:	USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL CELL CARCINOMA AND OTHER TUMORS
Examiner:	James Anderson
Group Art Unit:	1614

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DECLARATION OF DR. SINAN TAS

SINAN TAS states:

I. Background and Expertise

1. I am one of the inventors of the above titled application. I am a Medical Doctor (M.D. Hacettepe University Ankara, Turkey, 1976). I have a Ph.D. in Experimental Pathology (University of California at Los Angeles, 1980). I have also received additional educational certificates and diplomas detailed in my Curriculum Vitae, attached as **Exhibit A**.

2. My professional experience includes numerous clinical and teaching positions at academic institutions in the United States, Turkey and Kuwait working in the fields of pathology, biochemistry, clinical biochemistry, and molecular genetics. These positions are also listed in **Exhibit A**.

3. In the course of my work I have assisted in developing a first year medical curriculum at İnönü University, Malatya, Turkey and Ph.D. programs in oncology and

biochemistry at Dokuz Eylül University, Izmir, Turkey. I have also done extensive research in the fields of molecular and cellular mechanisms of aging, the structure and regulation of the eukaryotic genome and in tumor biology. I have numerous publications in those fields which are also listed in **Exhibit A**.

4. Based on my training and experience I have specific expertise in the molecular and cellular mechanisms of aging and of tumorigenesis and I am qualified to evaluate the state of the art discussed herein.

II. Level of Skill in the Art

A. Derivatives of Cyclopamine

5. Cyclopamine had long been known in prior art and identified originally due to its teratogenic effects. Teratogenicity of cyclopamine and of the related teratogenic Veratrum alkaloids had been found in prior art to arise from the inhibition of Hedgehog/Smoothed (Hh/Smo) signaling by them which resulted in the inhibition of differentiation of a group of cells in the developing embryo as a consequence (see references cited on page 3, lines 5-6 of specification). A wealth of information was publicly available in prior art about cyclopamine and about the related teratogenic compounds, including about their molecular structure, physical and chemical properties and about how to obtain them by purification from natural sources or through total chemical synthesis [see e.g. *Keeler RF, Teratology* 1970; 3:169-173 (**Exhibit B**); *Kutney JP et al, Canadian Journal of Chemistry* 1975;53:1796-1817 (**Exhibit C**); *Browne CA et al, Journal of Chromatography* 1984;336:211-220 (**Exhibit D**)]. Structure/function correlations (structure - activity relationships) were also described in prior art with the teratogenic Veratrum compounds as well as with compounds derived from them (see e.g. **Exhibit B** and *Beachy PA, WO 01/27135*, page 94, line 15 to page 98, line 22).

6. The synthesis of various derivatives of a pharmaceutically active molecule, as well as the generation of large libraries of related molecules based on modifications of the constituent structure, can be readily accomplished by modern methods of organic chemistry (e.g.

combinatorial chemistry). *Dudek et al*, US 6,291,516, recognizes this fact (see column 60, line 51 to column 61, line 42). The specification of present application indicates that molecules can be derived from cyclopamine or synthesised in such a way that they possess structural features to exert similar receptor binding properties and biological effects as cyclopamine. Such a molecule is said to be referred to as a "derivative of cyclopamine" in the specification and further information is provided about it (see page 10, lines 8-26). Such a compound is described to bind specifically to the same biological target as cyclopamine to exert the same biological effects as cyclopamine. Said effects, according to the specification, include the inhibition of Hh/Smo signaling and the causation of the technical effects recited in claim 1, when such a molecule is used according to the described method. Armed with such a clear functional definition, the skilled person can readily screen a huge number of molecules by looking for the described desirable functional effect. Assays for Hh/Smo signaling as well as for apoptosis and markers of differentiation were well known in art and widely available. High throughput versions of such assays, allowing screening together of large chemical libraries, were also known and used in prior art.

B. Tumor Treatment Methods

7. A person skilled in the art of tumor treatment has experience in routine clinical practice with conventional cancer drugs, which involves the adjustment of dosage according to the characteristics of tumor and of the patient and the expected effects of particular drugs. When informed of what effects a drug will produce, the skilled practitioner can readily optimize the administration of medicament for different tumors and patients. In this respect, the methods for determining the occurrence of apoptosis as well as of the markers of differentiation have been well known in art (they are commonly a part of the routine clinical laboratory practice). A decrease of size or disappearance of a tumor in response to an administration can also be accurately determined by the imaging methods routinely employed in clinical practice. What was not known, prior to our invention, was to look for tumors which utilize hedgehog/smoothened signaling for inhibition of tumor cell differentiation and for inhibition of tumor cell apoptosis and to develop dosages of cyclopamine or cyclopamine derivatives which go beyond inhibition of

proliferation, to achieve differentiation and apoptosis of the tumor cells. In summary, all of the methods needed to practice the invention were well known, except for the information of what effects were to be expected and therefore targeted for dosing.

III. Specification Enablement - Non-BCC Tumors

8. In an Office Action mailed September 11, 2006 in our US Patent application identified above, the Examiner has asserted that while the application is enabling for administration of cyclopamine to BCC (basal cell carcinoma) tumors, it is not enabling for inducing cellular differentiation and/or apoptosis in all tumors by administering cyclopamine. I wish to emphasise that the presently claimed method is not directed to any tumor or any tumor cell. Rather, it is directed to a specifically defined type of tumor, that is, a tumor in which Hh/Smo signaling is utilized for inhibition of tumor cell differentiation and/or for inhibition of tumor cell apoptosis. In my opinion the application does teach the skilled person how to make and use the invention for this class of tumors, without undue experimentation.

9. The specification of the present application describes a method of treatment for a specifically defined group of tumors and shows that it acts, unlike the conventional methods of drug and radiation treatments, by inductions of tumor cell differentiation and apoptosis in a highly selective, efficient and non-genotoxic manner. The specification describes and exemplifies successful applications of the method on several independent tumors (>7) and on more than one type of tumor that qualify for the specific definition given in claim 1. The method was successful each time it was applied and not only on BCC's but also on the other types of tumors within the defined category (e.g. trichoepithelioma and melanocytic tumors). All of the exemplified tumors were human tumors. The described examples are highly relevant to the practice of invention clinically. Actual human patients bearing the tumors were treated to attain the technical effects and therapeutic results that are described in specification. Microscopic views of the tissue sections are shown that show that the new method of treatment causes a "massive apoptotic death and removal of tumor cells" so as to cause "disappearance of tumor nodules to leave behind cystic spaces with no tumor cells." All of this is shown to be achieved

while sparing normal tissue cells (including those with stem and progenitor cell features). In addition to the clinical photographs and microscopic views showing the therapeutic results and technical effects, statistical analyses are also shown which document the reproducibility and efficiency of the inductions of tumor cell differentiation and apoptosis by the new method of treatment (see page 21, Table 1). The skilled person will realize that a statistical analysis of the described treatment results (successful outcome in each of >7 independent tumors) also shows a high predictability of the method on the specific type of tumor and tumor cells described in the pending claims.

10. A person skilled in the art is also guided by the specification in practicing the invention on other tumors that qualify as described in the pending claims. The demonstration that the qualified tumor cells can be caused to undergo differentiation and apoptosis *in vivo* with the described efficiency and selectivity, by administration of a sufficient dosage of medicament, is by itself highly informative to the skilled person. The specification teaches conditions providing the described technical effects with independent exemplified tumors (page 4, line 22 to page 6, line 9; page 7 line 1 to page 8, line 27; page 10 line 28 to page 14, line 2; page 19, line 4) and the skilled person is reminded that the optimal quantity and application schedule of medicament depends on the size and location of tumor as well as other factors that are well known in art.

11. The specification of our application describes that the use of cyclopamine and cyclopamine derivatives can be used in treatments of BCC (basal cell carcinoma) and non-BCC tumors so as to cause a rapid regression or disappearance of tumors that employ the Hh/Smo signaling for inhibition of apoptosis and/or for inhibition of differentiation of tumor cells. Information on specific non-BCC tumors is provided on page 7, line 21 to page 8, line 4; page 16 line 22 to page 17 line 4; page 17 lines 4-11; and Figures 6A, 6B, 6C, 6D. Further, the specification teaches that the results obtained with the exemplified tumors, including the sparing of normal stem and progenitor cells, indicate effectiveness in applications for internal tumors that also employ Hh/Smo signaling for inhibition of apoptosis and for inhibition of differentiation of tumor cells (e.g. the provision of guidance with regard to the doses when medicament is given by

systemic or intratumoral administration) (see page 11, lines 22-27; page 15, lines 19-24; page 19, lines 5-10; page 19, lines 12-29). Further relevant in this regard is the fact that patched 1 haploinsufficiency had been well recognized in prior art to cause predisposition to not only BCC's but also to other tumors in various organs and tissues of affected people. As noted in the specification (page 2, lines 13-16), this condition is called Nevoid Basal Cell Carcinoma Syndrome (NBCCS or Gorlin's syndrome). It was well known in prior art that, besides BCC's, occurrences of numerous other benign and malignant tumors (including medulloblastoma, fibroma, fibrosarcoma, meningioma, lymphoma, rhabdomyoma and schwannoma) are increased in the patients with NBCCS. For example, recurrent independent non-BCC tumors were described in many NBCCS patients and ovarian fibromas were found in 17% of the female NBCCS patients (*Kimonis VE et al, American Journal of Medical Genetics* 1997;69:299-308) (**Exhibit E**). Still further, the loss-of-function mutations of patched (i.e., the underlining cause of NBCCS) had been well known in prior art to cause upregulation of Hh/Smo signaling and were described also in the sporadic BCC's and various other sporadic tumors, including medulloblastoma, meningioma, breast cancer, uterine cervix and esophageal squamous cell carcinomas and trichoepithelioma [*Xie J et al, Cancer Research* 1997;57:2369-2372 (**Exhibit F**); *Vorechovsky I et al, Cancer Research* 1997;57:4677-4681 (**Exhibit G**); *Maesawa C et al, Genes Chromosomes Cancer* 1998;21:276-279 (**Exhibit H**); *Lu X et al, International Journal of Gynecological Cancer* 2000;10:452-458 (**Exhibit I**)]. Thus, a person skilled in the art, after reading the specification, understands readily that the described method can be utilized for treating not only BCC's but also other tumors that also qualify for the definition given therein.

12. Table 1, below, identifies teachings in our specification which indicate that the described method of treatment has utility in treatment not only of the BCC's but also of all those specific tumors that employ the Hh/Smo signaling for inhibition of apoptosis and/or for inhibition of differentiation of tumor cells.

Table I. Teachings In Specification That The Claimed Method Of Treatment Can Provide The Technical Effects Described In Claim 1 On The Non-BCC Tumor Cells That Also Employ The Hh/Smo Signaling For Inhibition Of Apoptosis And/Or For Inhibition Of Differentiation

<u>Page and Lines</u>	<u>Teaching</u>
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Page 1, lines 1-2	" ... treatment of basal cell carcinoma and other tumors "
Page 3, lines 24-28	" These novel effects ... make ... highly desirable in cancer therapy, in the treatment of BCC's and other tumors that use the hedgehog/smoothened signal transduction ... for ... prevention of apoptosis"
Page 4, lines 12-17	" In a further aspect, the invention is directed to the use of cyclopamine or ... a derivative of cyclopamine in the treatment of tumors that use the hedgehog/smoothened signal transduction ... for the prevention of apoptosis or ... differentiation "
Page 7, lines 21-25	" ... trichoepithelioma ... prior to treatment ... after 24 hours of treatment "
Page 7, line 27 to page 8, line 4	" ... section ... numerous apoptotic cells and the formation of cystic structures by their removal "
Page 9, line 22 to page 10, line 4	" ... tumor cells ... cyclopamine-induced differentiation ... apoptosis ... highly efficient and ... extreme specificity ... make it highly desirable in cancer therapy "
Page 11, lines 22-27	" Preservation of the undifferentiated cells ... provide information about the tolerable doses in other ... modes of administration ... intratumoral injection of an aqueous solution or systemic administration of the same or of cyclopamine entrapped in liposomes "
Page 15, lines 19-24	" ... highly efficient differentiation and apoptosis of the tumor cells in vivo ... at doses that preserve the undifferentiated tissue cells ... together with the non-genotoxic mode of action ... support the use ... not only on BCC's but also on those internal tumors that utilize the hedgehog/smoothened pathway for ... prevention of apoptosis and/or differentiation "
Page 16, line 22 to page 17, line 4	" Trichoepithelioma is another tumor associated with genetic changes that cause increased hedgehog/smoothened signalling ... rapid regression ... marked apoptotic activity ... cystic spaces resulting from the apoptotic removal of tumor cells "
Page 17, lines 4-11	" ... decreased size and pigmentation ... melanocytic tumor ... treatment ... in another volunteer also caused similarly rapid ... disappearance "
Page 19, lines 2-10	" ... tumor response to optimal concentrations ... was rapid ... examples illustrate effectiveness of the described treatment in the causations of tumor cell differentiation and apoptosis and in ... regression of the tumors displaying hedgehog/smoothened signaling. Effectiveness on several independent tumors in unrelated patients ... is consistent with the general utility "
Page 19, lines 12-29	" ... findings that a transient exposure to cyclopamine can suffice for the causations of tumor cell differentiation and apoptosis ... further ... facilitates treatment of internal tumors "
Page 22, line 1 to page 24 line 23	Originally filed claims 1-3, 5-14, 16-22 directed to a method employing cyclopamine or a pharmaceutically acceptable derivative thereof or to a medicament comprising the same for obtaining the technical effects described in claim 1 on the specific tumor cells defined therein.

13. Independent scientific investigations published after our invention confirm that cyclopamine or a derivative thereof can be utilized for obtaining the technical effects recited in claim 1 not only on BCC but also on the non-BCC tumor cells as described in claim 1. Such publications include *Berman DM et al, Science* 2002; 297:1559-1561 (**Exhibit J**), *Thayer SP et*

al, *Nature* 2003;425:851-856 (**Exhibit K**) and *Sanchez P et al*, *Mechanisms of Development* 2005;122:223-230 (**Exhibit L**). These published investigations include tumors located internally and a systemic mode of administration. The methods of treatment in these investigations are fully consistent with that described in the present application. For example *Berman DM et al* (**Exhibit J**), experimenting with medulloblastoma, describe inductions of tumor cell differentiation and apoptosis by use of cyclopamine and report that systemic administration of cyclopamine to tumor-bearing mice at a dosage of ~ 50 mg/kg body weight daily for 24 days resulted in the disappearance of established tumors. Similarly, *Thayer SP et al* (**Exhibit K**) describe that systemic administration of cyclopamine to the mice having pancreatic adenocarcinoma allograft at a dosage of ~ 48 mg/kg body weight daily for 7 days induced marked apoptotic death of tumor cells. In comparison, in the tumor treatment described by *Dudek et al*, who described an inhibition of tumor cell proliferation but did not describe induction of tumor cell apoptosis, the cyclopamine dosage given systemically to the medulloblastoma-bearing mice was magnitudes lower (1.1 mg/kg body weight daily for 14 days). In accordance, *Sanchez P et al* (**Exhibit L**) reported that systemic administration of cyclopamine to medulloblastoma-bearing mice at a dosage of 10 mg/kg body weight every other day for a month did not suffice to cause detectable tumor cell apoptosis although tumor cell proliferation was inhibited. They report further that cyclopamine can be used for inducing tumor cell apoptosis but the causation of this technical effect requires relatively greater doses than that is sufficient to inhibit tumor cell proliferation.

14. Regarding the assertion on pages 5-6 of the Office Action, that *Sanchez et al*, (**Exhibit L**), demonstrates that inducing differentiation and/or apoptosis is highly unpredictable, I do not agree that it would be viewed as such by a skilled person who has been given access to our application. *Sanchez et al*, confirms what is taught in the specification of present application, namely, induction of apoptosis of the tumor cells that are defined in claim 1 is dependent on the quantity and duration of application of cyclopamine. See page 228, second column: "Indeed, apoptosis driven by Hh pathway blockade is often more evident in vitro after a prolonged period of treatment or at high doses (data not shown)." It does not follow from this fact that the attainment of said technical effect on the specific tumor cells described in claim 1 is

unpredictable. Rather, it demonstrates that the application is correct in asserting that the dosage must be high enough to produce more than inhibition of proliferation.

15. Other scientific publications describing administration of cyclopamine onto tumor cells in vitro and the Hh/Smo signaling characteristics in various human tumors also confirm that the presently claimed method of treatment has utility in treatments of the specific tumors described in this application. Thus, *Mimeault M et al (International Journal of Cancer 2006; 118:1022-1031) (Exhibit M)* describe that cyclopamine can be utilized to induce apoptosis of the human prostate cancer cells in a concentration-dependent manner (see *e.g.* pg. 1025, col. 2).

16. *Sheng T et al, Molecular Cancer 2004;3:29 (Exhibit N)* provide further confirmation that the skilled person can identify non-BCC tumors encompassed within claim 1. Reporting findings with 59 human prostate cancer specimens, they describe that the prostate cancers that had a relatively greater proportion of tumor cells displaying features of differentiation had lower Hh/Smo signaling activity than the undifferentiated prostate cancers. Similar confirmatory evidence (i.e., the utilization of Hh/Smo signaling in inhibition of tumor cell differentiation) have been described independently with human uterine cervix squamous cell cancers and precursor lesions (*Xuan YH et al, Modern Pathology 2006;19:1139-1147) (Exhibit O)* and with ameloblastomas (*Kumamoto H et al, Journal of Oral Pathology and Medicine 2004;33:185-190) (Exhibit P)*. Also, *Oliver TG et al [Development 2005;132:2425-2439 (Exhibit Q)]* have described an analysis of the medulloblastoma development in the mice born, like the human NBCCS patients, with haploinsufficiency of patched 1. They have demonstrated that, whereas both the medulloblastoma cells and nontumorigenic granule cell precursors show proliferation, the tumor cells, but not the non-tumorigenic cells express the Hh/Smo signaling target genes whose products act to inhibit differentiation and apoptosis in tumor cells.

IV Non-obviousness over Dudek et al, US 6,291,516

17. I have read the disclosure of *Dudek et al* repeatedly and the comments regarding

this document in the Office Action. I do not agree that a skilled person, is led to the present invention by anything taught in this document. In this regard it is important to recognize that much of the disclosure in *Dudek et al* is so generalized and unsupported as to be useless. The patent purports to describe "regulators" of the hedgehog pathway in which an unlimited number of compounds are said to antagonize hedgehog signaling. In addition, another category of unlimited number of compounds are disclosed that may function to increase or decrease the intracellular cAMP concentration ¹. *Dudek et al* refers to all of these diverse compounds as "subject compounds", regardless of the fact that they have opposing regulatory actions. Further, a widely diverse range of conditions are indicated to be affected by these "regulators." Consequently, except when a clarification is made (or can be inferred) as to the nature of "subject compounds" to be used for a given condition, it is typically not clear which of the compounds are to be used for which condition and what regulatory effect may be expected.

18. Many of *Dudek et al*'s teachings lead away from the use of cyclopamine or another specific inhibitor of hedgehog signaling for induction of cellular differentiation. In Table II below, I have detailed teachings in *Dudek et al* which specifically would have lead away from use of cyclopamine or another specific inhibitor of hedgehog signaling for induction of differentiation.

Table II. Unambiguously Disclosed and/or Supported Teachings in *Dudek et al* That Teach Away From The Use Of Cyclopamine Or Another Specific Inhibitor Of Hedgehog Signaling For Induction Of Cellular Differentiation.

<u>Column and Lines</u>	<u>Teaching</u>
Col. 2, lines 5-7	"... critical inductive roles of hedgehog polypeptides in the development "
Col. 3, lines 16-17	" In the CNS, Shh ... induce ventral cell fates " (references are given)
Col. 3, lines 44-45	" ... Shh also induces the appropriate ventrolateral neuronal cell types " (references are given)
Col. 3, lines 60-61	" ... Shh mediates the induction of the sclerotome directly " (reference is given)

¹ Compounds that cause increase of intracellular cAMP are said to be used to inhibit hedgehog signaling. Whereas activation of the protein kinase A (PKA) by increased intracellular cAMP is known to be capable of inhibiting hedgehog signaling, it must be emphasized that the increases of intracellular cAMP and of PKA activity can have many other consequences in the cell besides the affection of hedgehog signaling. For example, many polypeptide hormones are known to act by causing elevation of intracellular cAMP. Further, the PKA activity is well known to affect Wnt/ β -catenin signaling besides hedgehog signaling. It is clear that the actions of the compounds disclosed to alter cAMP concentration would not be specific to hedgehog signaling.

Col. 42, lines 61-63	" Shh serves as an inductive signal ... which is critical "
Col. 43, lines 22-26	" ... differentiation of intestinal mesoderm ... depend on ... Sonic hedgehog " (reference is given)
Col. 48, lines 42-45	"... a subject compound can reduce the ... proliferation and differentiation of ... epithelium " (subject compounds that cause inhibition of proliferation are taught in <i>Dudek et al</i> to be compounds that inhibit hedgehog signaling)
Col. 49, lines 31-33	" lhh ... promote retinal progenitor proliferation and photoreceptor differentiation " (reference is given)
Col. 49, lines 34-39	"... treatment ... with ... Sonic hedgehog results ... in rod photoreceptors, amacrine cells and Muller glial cells " (reference is given)
Col. 67, lines 40-49	" Skin punches treated with ... Shh ... display ... follicle induction ... Shh leads to significantly increased induction "

19. *Dudek et al* teach also directly away from the use of subject compounds for induction of apoptosis. Referring to the requirement of proliferation for the induction of cell death by many agents used in treatment of cancer, *Dudek et al* disclose specifically that "*because a subject compound will often be cytostatic to epithelial cells, rather than cytotoxic*", subject compounds can be used to inhibit proliferation and to prevent thereby the programmed cell death in hair follicles (column 50, lines 20-29).

20. *Dudek et al* has no teaching that cyclopamine or another specific inhibitor of hedgehog signaling will induce apoptotic death and removal of tumor cells so as to cause a decrease of size or disappearance of tumor. Rather, *Dudek et al's* teachings point in the opposite direction, as elaborated below.

21. *Dudek et al* had actually evaluated cyclopamine as well as another specific inhibitor of hedgehog signaling (jervine) on the tumor cells that had constitutive activation of hedgehog signaling due to an inactivating mutation in the gene for patched. They describe the *in vitro* findings in column 63, lines 13-50 and in Figure 2 and disclose that both cyclopamine and jervine inhibited the proliferation of said tumor cells. No causation of cell death by cyclopamine or jervine is disclosed even though the treated cells were examined by microscopy.

22. Cyclopamine was evaluated by *Dudek et al* also *in vivo* on the same tumor (medulloblastoma) cells. The related experiments and findings are described in column 63, line 51 to column 64, line 22 and in Figure 3. Contrary to the mistaken statement on page 12 of the September 11, 2006 Office Action, *Dudek et al* do not describe, and the disclosed experiments and findings do not show, causation of a decrease of medulloblastoma tumor volume by the described cyclopamine treatment. *Dudek et al* describe that they established transplanted tumors in athymic mice by injecting medulloblastoma cells into their brain and by allowing 5 weeks for the tumors to grow. The tumor-bearing mice were then divided into two groups for treatment during the next 2 weeks with injections of cyclopamine (3 mice) or vehicle (2 mice). One of the three mice injected with cyclopamine was sickened during the course of injections and had to be killed before the end of 2 weeks. All remaining animals were killed at the end of the 2 weeks of treatment and the volumes of existing tumors were determined. Figure 3 shows the individual tumor volumes in arbitrary units (relative tumor volumes). The volume of the tumor from the cyclopamine-treated mouse that was killed earlier than the others is included inappropriately in the data set shown in Figure 3, with no indication of which data point belongs to said mouse. Whereas the cyclopamine-treated group had an average tumor volume smaller than in the vehicle-treated group, the difference was not significant ($p > 0.05$, calculated from the data set shown in Figure 3). No causation of cell death by the treatment with cyclopamine is disclosed even though serial sections of tumors were made and examined by microscopy. Such microscopic examinations ordinarily enable detection of apoptosis as shown for instance in the Figures of our application. In addition, design of the experiments described in *Dudek et al* is lacking a measurement of the volumes of growing tumors prior to the initiation of treatments (i.e., at the end of the 5th week). *Therefore, even in case of a significant difference between the vehicle and cyclopamine treated groups in terms of tumor volumes, these experiments would only permit a conclusion of an inhibition of tumor growth by the cyclopamine treatment.*

23. *Dudek et al* teaches that that a minimum dosage that will produce a therapeutic effect is to be used (column 54, lines 42-49; "... start ... at levels lower than that required ... and gradually increase the dosage until the desired effect is achieved"). What is contemplated in terms of tumor treatment is an inhibition of tumor growth (see column 64, lines 10-12), and what

is shown in *Dudek et al* in terms of the technical effect obtained on tumor cells by use of cyclopamine is an inhibition of proliferation. Thus the manipulations in the *Dudek et al* method involve an administration in a quantity that will produce the technical effect of the inhibition of tumor cell proliferation, but not more. It is specifically taught in *Dudek et al* not to administer beyond that quantity.

24. Our application describes a clear dose-response effect in the attainment of differentiation and/or apoptosis and emphasizes that a suboptimal dosing would not provide said technical effects (see e.g. page 18 line 21 to page 19 line 4 and Figure 7F).

25. Some of the independent investigations already mentioned which confirm the scope of the invention also report that tumor cell apoptosis is not obtained until a threshold dosage is exceeded that is above the dosage sufficient to inhibit tumor cell proliferation. Thus, *Sanchez P et al* (**Exhibit L**) describe that an amount or dosing of cyclopamine that caused inhibition of tumor cell proliferation did not cause a detectable tumor cell apoptosis and that the causation of latter technical effect required relatively higher doses. Similarly, *Mimeault et al* (**Exhibit M**) found with cell lines of human prostate cancer cells that induction of tumor cell apoptosis requires relatively greater amounts of cyclopamine and longer durations of treatment than needed to obtain an inhibition of proliferation of the same tumor cells. For example, it is described that treatment of the LNCaP-C33 prostate cancer cells with 2 μ M cyclopamine for two days caused inhibition of tumor cell proliferation (see Fig. 2 of **Exhibit M**) but even a treatment with more than twice of that amount for four days failed to cause detectable apoptosis (**Exhibit M**, Fig. 4A, 5 μ M Cycl). Apoptosis, however, is very clearly induced after treatment of said cells with 20 μ M cyclopamine for four days (**Exhibit M**, Fig. 4A, 20 μ M Cycl).

26. Besides the above mentioned evidence and facts, it should be noted that the design of the investigations described in the specification of this application entails medicament applications onto a part of tumor in a patient, followed by the analyses of responses in entire tumor. In such an approach, the tumor area that is adjacent to the area onto which cyclopamine is applied receives a relatively lower dosage of cyclopamine by diffusion from the area of direct

application. Thus, the effects of differing amounts of cyclopamine on tumor cells can be assessed with the cells of same tumor *in vivo*. The specification, in this respect, refers to the results of such investigations and emphasizes that a suboptimal dosing would fail to provide the technical effects described (i.e., the inductions of tumor cell differentiation and apoptosis; see e.g. page 18, line 21 to page 19, line 4). The tumor depicted in Figures 5A-F of specification is a tumor treated in such a manner and shows most directly that a dosage that suffices to inhibit tumor cell proliferation *in vivo* in a patient does not suffice to induce tumor cell apoptosis. The tumor of Figure 5A was treated by topical application of cyclopamine on half of its surface (the lower half in figure) whereas the other half (upper in figure) was left untreated. After treatment (Figure 5B) the entire tumor was removed and sectioned. Figures 5C-5F of the application depict various portions of the tumor stained by H&E or immunohistochemically for Ki-67 as described therein. Sections stained for Ki-67 are reproduced in color below as Figures 1A and 1B, at a relatively lower magnification than in the Figs 5E and 5F of application in order to show more tumor depth.

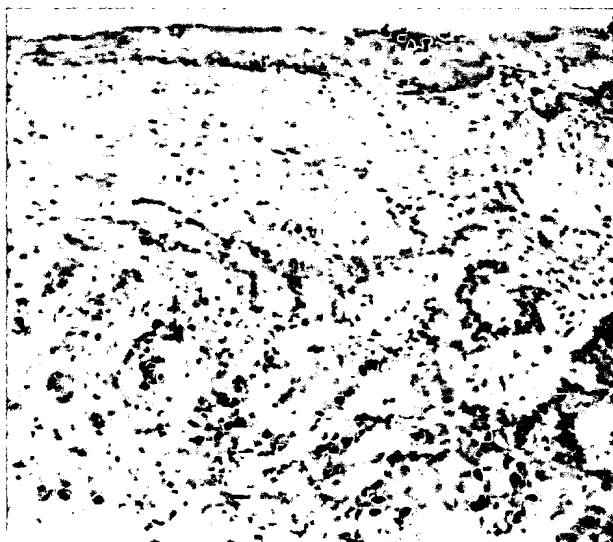


Figure 1A

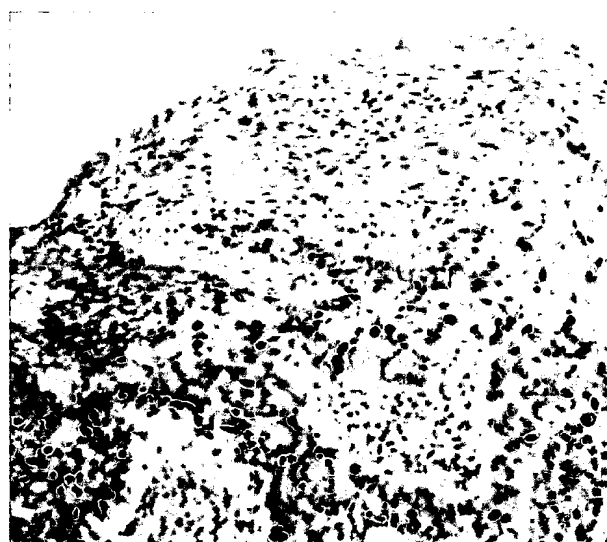


Figure 1B

Figure 1A: Section from the basal cell carcinoma shown in Fig. 5B of the specification. This section is from the half of tumor onto which the medicament was applied directly as described in the

specification. Immunohistochemical staining for Ki-67 (which is known to be a marker of proliferating cells) was done as described in the specification. The brown-labeled nuclei represent the proliferating cells (100 X original magnification).

Figure 1B: Section from the basal cell carcinoma shown on Fig. 5B of the specification. This section is from the half of tumor onto which cyclopamine was not applied directly. However, because of being in close proximity, a relatively lower concentration of cyclopamine than that in the adjacent area of direct application would reach to this area by diffusion from the area of direct application. Ki-67 staining (100 X original magnification).

27. Comparison of Figures 1A and 1B above shows that a relatively low dose of cyclopamine that does not exceed what is sufficient to inhibit tumor cell proliferation would fail to provide tumor cell apoptosis. The brown labeled tumor cell nuclei represent the proliferating tumor cells. In both Figures 1A and 1B, orientation of the tumor section is such that the tumor surface is towards the upper part of figure whereas the bottom of figure shows deeper regions of tumor. In both figures, the regions of tumor near the tumor surface show lack of Ki-67 labeling (i.e., the cells show inhibition of tumor cell proliferation) due to the cyclopamine that diffused down from the surface where it was applied directly (Figure 1A) or from the adjacent direct application area (Figure 1B). The deeper tumor regions show proliferating (Ki-67 labeled) tumor cells but the frequency of labeled tumor cells is relatively higher in the deeper tumor regions in Figure 1B than in Figure 1A. Notice in Figure 1A that the region of tumor near surface shows extensive formation of cystic spaces within the tumor. This is because the tumor cells therein were killed by apoptosis and removed to leave behind (empty) cystic spaces. By contrast, in Figure 1B the cell density is much higher in the tumor region towards the surface. In addition, the large cystic spaces that are seen in Figure 1A within the tumor are not visible in Figure 1B in the region where tumor cell proliferation was inhibited. Tissue sections that are stained for Ki-67 by immunohistochemistry are not suitable for quantification of apoptosis but H&E stained tumor sections are known to allow identification of the tumor cells having morphological features of apoptosis. For example, the H&E stained section in Fig 5C of specification shows numerous readily identifiable apoptosing tumor cells and demonstrates that the cystic spaces seen in the tumor are being formed as a result of the apoptotic death and removal of tumor cells. Counting apoptosing tumor cells in a microscopic field of an H&E stained section together with the total number of tumor cells allow one to obtain a measure of the frequency of apoptosing tumor cells therein. Said measurements can be repeated in other randomly sampled microscopic fields

belonging to a particular region of tumor so as to obtain a mean and a standard error of mean. Similar measurements of the Ki-67 labeling frequency in serial tumor sections (one stained for Ki-67 and next by H&E) thus allow one to measure the frequencies of proliferating and apoptosing tumor cells in a given region of tumor. This would provide an insight into the relative effects of the same concentration of cyclopamine on the proliferation and survival/apoptosis of tumor cells in the same tumor region *in vivo*.

28. I have done a quantitative comparison of the tumor cells showing proliferation and of the tumor cells showing apoptosis at three depths in the tumor shown in Fig 5B of specification. Graphic representation of my results are shown below in Figures 2A and 2B.

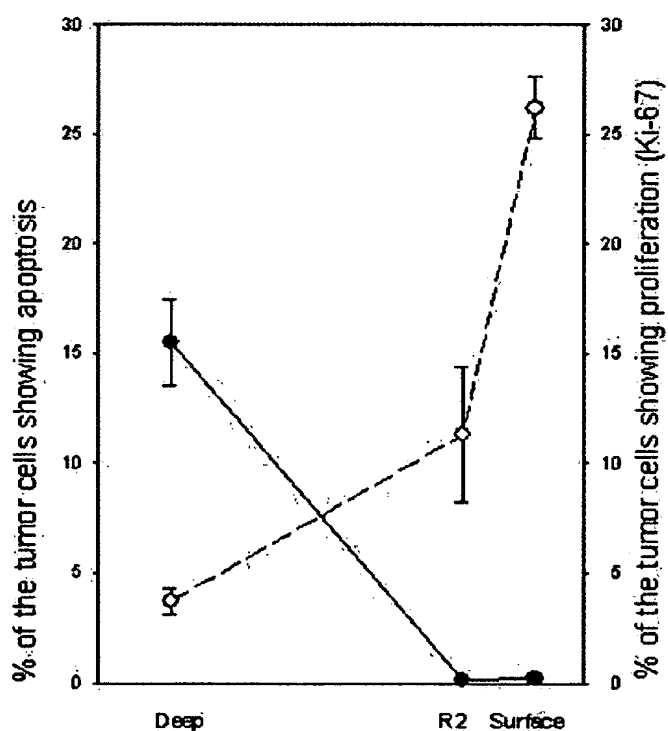


Figure 2A

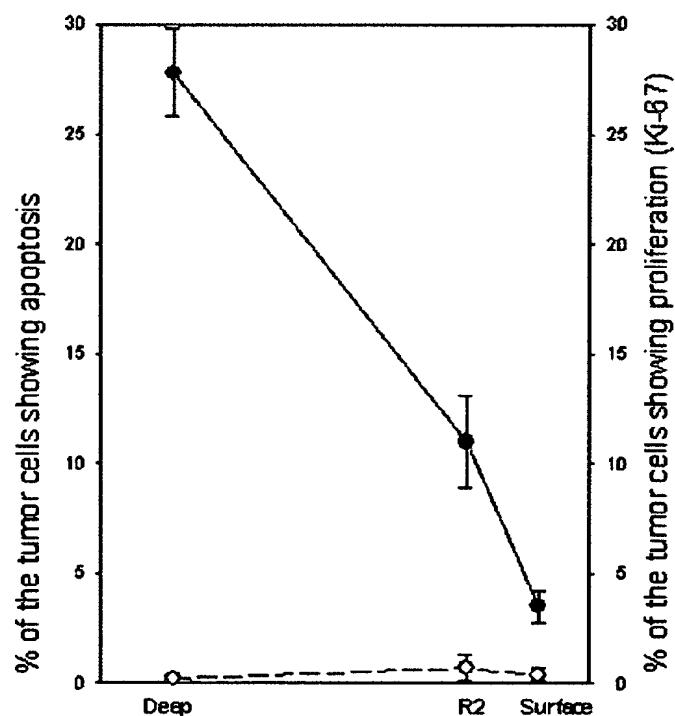


Figure 2B

Figures 2A and 2B show quantitative data concerning the frequencies of the proliferating tumor cells as well as of the apoptosing tumor cells in the different regions of the tumor shown in Fig. 5B of specification. Figure 2A shows data from the half of tumor where cyclopamine was applied directly. Figure 2B shows data from the half of tumor where cyclopamine could reach by diffusion from the adjacent direct application area. On the X axis, "Deep" refers to the region of tumor deep in dermis,

near the invading border. "Surface" refers to the surface region of tumor (i.e., the region that would receive the highest concentration of cyclopamine). "R2" refers to the region adjacent to the surface region. The scale on the X axis corresponds to the position of the analyzed regions in terms of depth from the tumor surface. The symbols (• — •) represent the frequency of Ki-67 labeled (i.e., proliferating) tumor cells. The symbols (o ... o) represent the frequency of tumor cells showing morphological signs of apoptosis.

The data in Figure 2A are from the half of tumor onto which cyclopamine was applied directly. The data in Figure 2B are from the half of tumor onto which cyclopamine was not applied directly; a relatively lower concentration of cyclopamine would have reached to this half by diffusion from the adjacent half onto which cyclopamine was applied directly. In addition, in both Figures 2A and 2B, the tumor cells near the surface (labeled as "Surface" on the horizontal axis in graphs) would have received greater dosage of cyclopamine than those tumor cells located deep in tumor (labeled as "Deep" on the horizontal axis in graphs). On the graphs, the symbols (• — •) represent the frequency of proliferating tumor cells and the symbols (o o) represent the frequency of tumor cells showing features of apoptosis (the bars on the two sides of symbols represent the standard errors). The scale on the horizontal axis corresponds to the position of analyzed regions in terms of depth from surface. Notice that the highest frequency of proliferating tumor cells was found in the tumor region ("Deep" in Figure 2B) that is furthest away from the site of cyclopamine application. Notice also that relatively higher doses of cyclopamine are required for the induction of tumor cell apoptosis than that would be sufficient to inhibit tumor cell proliferation. These quantitative data demonstrate clearly that stopping administration of cyclopamine at a dosage that suffices to inhibit tumor cell proliferation would not produce detectable apoptosis of tumor cells. Hence, a treatment as described by *Dudek et al* would not be able to provide detectable tumor cell apoptosis or the disappearance of an existing tumor.

29. Cyclopamine or a derivative thereof or another specific inhibitor of Hh/Smo signaling had been applied in prior art on tumor cells and on transformed cells displaying constitutive activation of Hh/Smo signaling. Table III, below, shows the prior art documents reporting results of said investigations along with their relevant teachings and show that they were leading away from the present invention. These prior art documents were previously filed in IDS submissions as indicated in the Table.

Table III. Teachings In Prior Art That Teach Away From The Use Of Cyclopamine Or A Derivative Thereof For Inducing Tumor Cell Apoptosis

Document	Filed date of IDS (document no.)	Teaching
Dunn MK et al, <i>Developmental Dynamics</i> 1995;202:255-270	5/3/06 (CAC)	" ... cell death is a specific effect of cyclopamine on (normal) cranial crest cells ... viability ... of cell line 3T3L1 or ... PC12 " are not affected (page 259, left column, 3 rd paragraph). " ... 3T3L1 and PC12 cell lines are resistant to cyclopamine-induced cell death " (page 265, right column, 2 nd paragraph). See also the Abstract and Fig. 12 (page 267) (PC12 is a well known tumor-derived and tumorigenic cell line).
WO 99/52534	1/27/04 (BA)	Treatment of patched -/- cells displaying constitutive activation of hedgehog signaling with cyclopamine caused marked inhibition of said signaling but <i>did not cause cell death</i> (page 102, lines 23-30). " ... because a hedgehog antagonist will often be cytostatic to epithelial cells rather than cytotoxic ", subject compounds can be used to inhibit proliferation and to prevent thereby the programmed death of cells (page 54, lines 1-11).
WO 01/27135	1/27/04 (BD)	Treatment of patched -/- cells displaying constitutive activation of hedgehog signaling with cyclopamine or KAAD-cyclopamine (a derivative of cyclopamine) did not cause cell death " ... even at the highest doses of compounds used " that caused inhibitions of the hedgehog signaling and of cellular proliferation. " ... because a subject compound will often be cytostatic to epithelial cells, rather than cytotoxic ", such agents can be used to inhibit proliferation and to prevent thereby the programmed death of cells (page 68, lines 11-21).
Taipale J et al, <i>Nature</i> 2000;406:1005-1009	1/27/04 (CC)	Treatment of patched -/- cells displaying constitutive activation of hedgehog signaling with cyclopamine or with KAAD-cyclopamine (a derivative of cyclopamine) caused dose-dependent inhibitions of both hedgehog signaling and of cellular proliferation but did not cause cell death (page 1007, Figure 3, legend).

30. It is clear from the documents mentioned in Table III above that cyclopamine and other specific inhibitors of hedgehog signaling were actually administered in prior art on tumor cells and on the transformed cells displaying constitutive activation of hedgehog signaling, but not to an extent that apoptosis was induced. Descriptions of none of these investigations included an induction of apoptosis; to the contrary, it was mentioned specifically that the treatments causing a complete inhibition of hedgehog signaling as well as marked inhibition of proliferation did not cause a death of the transformed and tumorigenic cells. Thus, the experimental findings described in the prior art with regard to the effects on tumor cell survival/death are simply the opposite of what is described in this invention. Not only the use of

an inhibitor of hedgehog signaling for inducing tumor cell apoptosis is surprising, but also the sparing of normal cells (particularly the normal stem and progenitor cells) under conditions causing a massive apoptosis of tumor cells is highly unexpected. *Thus, the skilled person would not even contemplate trying to find conditions where the technical effects described in our invention would be achieved.*

31. Prior to our invention, there were also other teachings in the prior art that would have led the skilled person away from the present invention. In particular:

a) Induction of tumor cell apoptosis *in vivo* in a selective and efficient manner so as to cause disappearance of tumors while sparing normal cells was considered in prior art as an unattainable goal on theoretical grounds and in view of the vast clinical experience (see e.g. the review article "*Apoptosis and the dilemma of cancer chemotherapy*"; Hannun YA, *Blood* 1997;89:1845-1853 [Exhibit R]).

b) A well known problem of conventional non-surgical treatments of cancer is the causation of damage to normal stem cells. Yet, these cells were described in prior art to be absolutely dependent on hedgehog signaling for their maintenance (Zhang Y et al, *Nature* 2001;410:599-604 [Exhibit S]).

c) Hedgehog proteins have been well known as inducers of differentiation of various cell types and the experiments where hedgehog signaling was inhibited by specific inhibitor compounds had shown inhibition of differentiation by said compounds (see e.g. the references mentioned in Table II).

d) Description of cyclopamine in prior art as a teratogen would discourage its consideration as a medicament.

32. The previously unknown technical effects of the inductions of tumor cell differentiation and apoptosis by use of a specific inhibitor of hedgehog signaling, with the efficiency and selectivity described in this invention, provided the therapeutic outcome of rapid regression and disappearance of tumors while sparing normal cells. The normal cells having the attributes of stem or progenitor cells, which are well known to be hit by most conventional cancer treatments to such an extent to limit the applicability and efficacy of such treatments, are

shown to be spared. These described features of present method clearly distinguish it from prior art methods of tumor treatment and makes it superior to them. Non-limiting examples of the clinical conditions where the presently described method of tumor treatment would be preferred over prior art methods include the following.

a) Rapid shrinking and disappearance of tumors, owing to the induction of a massive apoptotic death and removal of tumor cells, identifies a group of patients in whom surgical removal of tumor may not be feasible because of the location and/or size of tumor and in whom a mere inhibition of further tumor growth may be insufficient. Treatment of such a patient may be attempted by conventional means, e.g. by irradiation of tumor, but this is well known to be unfeasible or inadvisable for particular tumors and patients.

b) Tumors are known to undergo decrease of size *in vivo* for reasons other than the induction of tumor cell apoptosis, for example due to the causation of necrosis. However, removal of tumor cells by apoptosis is preferred for several reasons, including the severe inflammatory reaction that follows necrosis but not apoptosis.

c) Cessation of cellular proliferation, when it occurs because of being coupled to a certain differentiation state, would be advantageous in tumor treatment over a treatment protocol involving inhibition of tumor cell proliferation with little or no induction of differentiation. Many prior art methods of treatment belong to this latter category and the need for continuous administration of medicament for inhibition of tumor cell proliferation poses a dilemma because of the toxicities that become apparent with such administration. Cessation of tumor cell proliferation after an initial treatment, as described in this application, would provide a distinct advantage.

33. The undersigned Declarant having been warned that willful false statements and the like are punishable by fine or imprisonment, or both under the provisions of 18 U.S.C. §1001 and may jeopardize the validity of the application or any patent issuing thereon, hereby declares that all statements made of the Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

July 17, 2009
Date

Sinan Tas
Sinan Tas
Declarant

In Re Application of: Sinan Tas
Application No: 10/682584
Filed: October 9, 2003
For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit A

Curriculum Vitae

CURRICULUM VITAE

Name : Sinan Taş
Date of Birth : 14 March 1951
Place of Birth : Çukurkuyu, Turkey
Citizenship : Turkish
Marital Status : Single
Address for Correspondence : S. H. Tuncel Caddesi No. 36,
Bor, 51700 Turkey.

Facsimile : + 90. 388. 3131250
Telephone : + 90. 388. 3131599

Education and Training :

<u>Institution and Location</u>	<u>Degree or Certificate</u>	<u>Year Conferred</u>	<u>Scientific Field</u>	<u>Year Entered</u>
Hacettepe University Faculty of Medicine, Ankara, Turkey	License	1972	Biological and Medical Sciences	1969
Hacettepe University Faculty of Medicine, Ankara, Turkey.	M.D.	1976	Medicine	1969
University of California at Los Angeles, CA, U.S.A.	Ph.D.	1980	Experimental Pathology	1976
University of Texas M.D Anderson Hospital and Tumor Institute, Houston, TX, U.S.A.	Fellowship Certificate	1982	Laboratory Medicine	1980

Special Diplomas :

Permanent Certificate of the Educational Council for Foreign Medical Graduates (U.S.A.)

Board Certificate in Clinical Biochemistry, Ministry of Health (Turkey, 1982)

"University Doçent" Certificate in Biochemistry-Clinical Biochemistry, The Inter-Universities Council (Turkey 1984)

Professional Experience :

<u>Position</u>	<u>Department</u>	<u>Employer</u>	<u>Period</u>
Fellow	Laboratory Medicine	University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, U.S.A.	1980 - 1982
Clinical Biochemist, Lieutenant	Clinical Biochemistry	Turkish Armed Forces Gülhane Military Medical School Hospital, Ankara, Turkey.	1982 - 1983
Chief Research Scientist	Research Institute for Basic Sciences	Scientific and Technical Research Council of Turkey.	1983 - 1985
Seconded Faculty (Doçent)	Biochemistry and Clinical Biochemistry	Marmara University Faculty of Medicine Istanbul, Turkey.	1984 - 1985
A. Professor	Pathology	Kuwait University Faculty of Medicine, Kuwait.	1985 - 1991
Consultant	Clinical Biochemistry Laboratories	Mubarak Al-Kabeer Teaching Hospital, Ministry of Public Health, Kuwait.	1985 - 1991
Consultant	MRCPath Program	Kuwait Institute for Medical Specialization, Ministry of Public Health, Kuwait.	1987 - 1991
Professor	Biochemistry and Clinical Biochemistry	İnönü University Faculty of Medicine, Malatya, Turkey.	1992 - 1994
Director	Clinical Chemistry Lab.	İnönü University Hospital, Malatya, Turkey.	1992 - 1994
Professor	Biochemistry and Mol. Genetics	Dokuz Eylül University Faculty of Medicine and Institute of Oncology, İzmir, Turkey.	1994 - 2003
Scientist		İleri Health and Life Sciences Research, Manufacturing, Service and Trading A.Ş., Turkey.	2003 - Present

Teaching Experience :

<u>Position</u>	<u>Subjects</u>	<u>Institution and Location</u>	<u>Period</u>
Doçent (seconded)	Biochemistry, Molecular Genetics	Marmara University, Faculty of Medicine, Istanbul, Turkey.	1984-1985
A. Professor	Clinical Chemistry, Molecular Pathology	Kuwait University Faculty of Medicine, Kuwait.	1985-1991
A. Professor, Supervisor	Clinical Chemistry, Molecular Pathology, Molecular Genetics, (MSc Progr., Pathology)	Kuwait University College of Graduate Studies, Kuwait.	1987-1991
Consultant	Clinical Chemistry, (MRCPath Program)	Kuwait Institute for Medical Specialization, Ministry of Public Health, Kuwait.	1987-1991
Professor	Biochemistry, Clinical Chemistry, Mol. Genetics	İnönü University Faculty of Medicine, Malatya, Turkey.	1992-1994
Professor	Biochemistry, Clin. Chemistry Mol. Genetics and Pathology	Dokuz Eylül University Faculty of Medicine and Institute of Oncology, İzmir, Turkey.	1994-2003

Courses and Curricula Developed :

Integrated first year medical school curriculum (chairman of committee), İnönü University Faculty of Medicine, Malatya, Turkey (1993)

Ph.D. program in oncology (biochemistry and molecular genetics; chairman of committee), Dokuz Eylül University Institute of Oncology, İzmir, Turkey (1995)

Ph.D. program in biochemistry (member of committee), Dokuz Eylül University Faculty of Medicine, İzmir, Turkey (1998)

Supervision of Theses :

Rajender Sandella Kumar, MSc (Ireland)
Reema Ramadan Zein El Din, MSc (Kuwait)

Administrative Responsibilities :

Chairman, Department of Pathology Research Committee, Kuwait University, Kuwait (1985-1991)

Scientific Program Secretary and Member of the Organizing Committee, International Workshop of Kuwait University "Molecular Genetics In Studies of Human Disease", Kuwait (1988)

Chairman, Department of Biochemistry, Faculty of Medicine, İnönü University, Malatya, Turkey (1992-1994)

Director, Clinical Chemistry Laboratory, İnönü University Hospital, Malatya, Turkey (1992-1994)

Member of the Biochemistry and Clinical Biochemistry Doçent Evaluation/Examination Committees of the Inter-Universities Council of Turkey (1993-2003)

Primary Research Interest : Molecular and cellular mechanisms of aging
Structure and regulation of the eukaryotic genome

Funded Research :

<u>Research Project</u>	<u>Period</u>	<u>Funding Agency</u>
"Study and Diagnosis of the Human Diseases With Primary Genetic Components by Molecular Genetic Techniques" (MG 016), Principal Investigator.	1986-1988	Kuwait University, Kuwait.
"Influence of Apolipoprotein-CIII Gene Polymorphism on the Metabolism of Plasma Lipoproteins and of Glucose" (RA 077), Principal Investigator.	1988-1989	Kuwait University, Kuwait.
"Computer-Aided Nucleic Acid and Protein Structure Analyses" (RA 031), Principal Investigator.	1989-1990	Kuwait University, Kuwait.
"Study of Telomere Maintenance Mechanisms In Normal and Tumor Cells", Principal Investigator [funding approved but not implemented owing to the Turkish university system's shortcomings (cf. <i>Turkish Journal of Biochemistry</i> 1998; 23:42-47); research efforts while in DEU were then carried out by own funds]	1994-	Dokuz Eylül University, Turkey.

Scientific Publications

Refereed Papers

1. Taş S. and Avcı O.: Induction of the differentiation and apoptosis of tumor cells in vivo with efficiency and selectivity. *European Journal of Dermatology* 2004;14:96-102.
2. Taş S. and Avcı O.: Rapid clearance of psoriatic skin lesions induced by topical cyclopamine. *Dermatology* 2004;209:126-131.
3. Taş S. and Abdella N.A.: Blood pressure coronary artery disease and glycaemic control in type 2 diabetes mellitus: relation to apolipoprotein-CIII gene polymorphism. *Lancet* 1994;343:1194-1195.
4. Taş S.: Genetic predisposition to coronary heart disease and the gene for apolipoprotein-CIII. *Lancet* 1991;337:113-114.
5. Taş S. and Zein El Din R.R.: Automated fructosamine assay with improved accuracy used to quantify nonenzymatic glycosylation of serum proteins in diabetes mellitus and chronic renal failure. *Clinical Chemistry* 1990;36:1825-1830.
6. Taş S.: Purification of DNA from clotted blood. *Clinical Chemistry* 1990;36:1851.
7. Taş S.: Separation of the DNA molecules beyond conventional size limits by gel electrophoresis with sodium dodecyl sulfate. *Analytical Biochemistry* 1990;188:33-37.
8. Taş S.: Molecular genetics in the study of human disease. *Medical Principles and Practice* 1989;1:174-176.
9. Taş S.: Strong association of a single nucleotide substitution in the 3'-untranslated region of the apolipoprotein-CIII gene with common hypertriglyceridemia in Arabs. *Clinical Chemistry* 1989;35:256-259.
10. Taş S., De Larco J. and Altıok E.: Agarose gel electrophoretic evidence for domains of nuclear DNA linked with bonds cleavable with sulfhydryl molecules. *FEBS Letters* 1985;191:136-140.
11. Taş S., Drewinko B., Rodriguez L.V. and Trujillo J.M.: Consistently greater decondensation of the nuclear DNA-protein complexes from normal lymphocytes than from acute and chronic lymphocytic leukemia cells following treatment with disulfide reducing agents. *Cytologia* 1985;50:405-415.
12. Taş S., Rodriguez L.V., Drewinko B. and Trujillo J.M.: Evidence for extensive intermolecular disulfide bonds of the proteins of nonionic detergent-high salt-resistant skeletons of normal lymphocytes and the altered structure in leukemia. *International Journal of Cancer* 1984;34:329-333.
13. Taş S. and Lichtiger B.: Preservation of a near neutral pH in human platelet concentrates stored at 22 °C. *Sangre* 1982;28:221-224.
14. Taş S. and Walford R.L.: Influence of disulfide reducing agents on the fractionation of chromatin complex by endogenous nucleases and DNase I in aging mice. *Journal of Gerontology* 1982;37:673-679.
15. Taş S. and Walford R.L.: Increased disulfide mediated condensation of the nuclear DNA-protein complex in lymphocytes during postnatal development and aging. *Mechanisms of Ageing and Development* 1982;19:73-84.
16. Taş S., Tam C.F. and Walford R.L.: Disulfide bonds and the structure of the chromatin complex in relation to aging. *Mechanisms of Ageing and Development* 1980;12:65-80.
17. Taş S.: Involvement of disulfide bonds in the condensed structure of facultative heterochromatin and implications on cellular differentiation and aging. *Gerontology* 1978;24:358-364.

18. Taş S.: Note on the Orgel hypothesis and on the mechanisms of aging. *Gerontology* 1977;23:306-308.
19. Taş S.: Disulfide bonding in chromatin proteins with age and a suggested mechanism for aging and neoplasia. *Experimental Gerontology* 1976;11:17-24.

Book Chapter

Taş S.: Cellular aging, neoplastic transformation, meiotic rejuvenation and the structure of chromatin complex. In Sauer H.W. (Ed.) *Cellular Ageing*, Karger, Basel, 1984, pp.178-192.

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1. Taş S.: Mechanism-based treatment of tumors. *Melanoma Research* 2006;16:S99.
2. Taş S. and Zein El Din R.R.: Aging is associated with elevation of nonenzymatically glycosylated proteins in serum. *Clinical Chemistry* 1990;36:1164.
3. Taş S. and Zein El Din R.R.: Nonenzymatically glycosylated serum proteins quantitated by a modified automated fructosamine assay with improved accuracy. *Clinical Chemistry* 1990;36:1157.
4. Taş S.: Greatly increased electrophoretic mobility of DNA in gels in the presence of sodium dodecyl sulfate. *Clinical Chemistry* 1990;36:1016.
5. Abdella N.A., Taş S., Zein El Din R.R. and Salman A.D.: Relationship of the nonenzymatic glycosylation of serum proteins to other clinical and laboratory findings in type II diabetes mellitus. *Fourth Kuwait International Medical Sciences Conference: Diabetes Mellitus*. Kuwait, 1990, p.S4.7.
6. Taş S.: Elevation of the very low density lipoproteins in plasma associated with a single nucleotide substitution in the 3'-untranslated region of the apolipoprotein-CIII gene. In Cleve H. (ed.) *Proceedings of the Society for Human Genetics*, Munich, 1989, p.69.
7. Taş S.: Age-related chromatin changes. In Doria G and Weksler, M.E. (eds.) *Ageing, Immune Response and Biological Modifiers*. Istituto Scientifico Roussel, Milano, 1986, p.24.
8. Taş S., Drewinko B. and Trujillo J.M.: Disulfide mediated condensation of the nuclear DNA-protein complex (DPC) in normal and leukemic lymphocytes. *Proceedings of the American Association for Cancer Research*, 1982;23:11.
9. Taş S. and Walford R.L.: Disulfide bonds and the structure of the chromatin complex in relation to aging and spontaneous malignancies of old age. *Age* 1980;3:95.
10. Taş S. Tam C.F and Walford R.L.: Role of disulfide bonds in chromatin structure. Influence of aging. *Gerontologist Part II* 1978;18:132
11. Taş S. and Walford R.L.: Possible role of disulfide bonds in chromatin in differentiation, meiotic rejuvenation and aging. *Gerontologist Part II* 1978;18:131.
12. Taş S. and Walford R.L.: Age related changes in hyperchromicity of DNA in chromatin under the influence of alkaline pH. *Gerontologist Part II* 1978;18:131.

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1. Taş S. Shortcomings of the clinical laboratory services in Turkey. Proposal for improvement (in Turkish). *Türk Biyokimya Dergisi/Turkish Journal of Biochemistry* 1998;23:42-47.
2. Taş S. Nucleic acid probes in the study and diagnosis of disease. *Clinical Biochemistry Newsletter* 1987;3:6-31.

3. Taş S. Generation of hybridomas secreting monoclonal antibodies. *TUBITAK Bulletin*, 1985, No.5.

Patent Files :

1. Taş S. and Avcı O.: Use of cyclopamine in the treatment of basal cell carcinoma and other tumors. 02 July 2001, PCT/TR 01/00027. *PCT Gazette*, 10 October 2002, WO 02/078703 A1 [republished on 12 June 2003 for correction of the publication error of *WIPO (PCT Gazette No. 24/2003 of 12 June 2003, Section II)* ; available at the *World Intellectual Property Organization* web site, www.wipo.int].
2. Taş S. and Avcı O.: Use of cyclopamine in the treatment of psoriasis. 19 April 2002, PCT/TR 02/00017. *PCT Gazette*, 10 October 2002, WO 02/078704 A1 (available at the *World Intellectual Property Organization* web site, www.wipo.int).
3. Taş S. and Avcı O.: Use of cyclopamine in the treatment of psoriasis and other skin diseases. 17 March 2003, PCT/TR 03/00017. *PCT Gazette*, 30 October 2003, WO 03/088964 A1 (available at the *World Intellectual Property Organization* web site, www.wipo.int).

In Re Application of: Sinan Tas
Application No: 10/682584
Filed: October 9, 2003
For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit B

Keeler RF, *Teratology* 1970;3:169-173.

Teratogenic Compounds in *Veratrum californicum* (Durand)

IX. STRUCTURE-ACTIVITY RELATION

RICHARD F. KEELER

U. S. Department of Agriculture, Agricultural Research Service,
Animal Disease and Parasite Research Division,
1150 East 14th North, Logan, Utah 84321

ABSTRACT The structures and configurations of three naturally occurring steroidal alkaloid teratogens from *Veratrum californicum* that induce cyclopia in sheep have been elucidated. The probable teratogenic structural requirements were examined in light of known structural and configurational information on active and nonactive compounds. Methods are proposed that will enable elucidation of the precise structural requirements for this teratogenic effect.

Veratrum californicum is a plant of ubiquitous distribution in high wet meadows of the Rockies, Sierras, and other mountain ranges of northern latitudes of North America. Ingestion of the plant by sheep on the 14th day of gestation gives rise to cyclopia and related cephalic malformations in offspring (Binns et al., '63, '68). Recent studies demonstrated that the teratogenic effect can be induced by each of three alkaloids of the plant (Keeler and Binns, '68; Keeler, '69a) whose structures are now known (Keeler, '69b,c). This information along with that available (Keeler and Binns, '68) on compounds of related structure not producing cyclopia allow an analysis of the probable structural specificity for this teratogenic effect. The analysis and investigation routes for further clarification are the subject of this report.

The three naturally occurring teratogens are closely related steroidal alkaloids — jervine, cyclopamine (previously labeled alkaloid V), and cycloposine (alkaloid X) (fig. 1). Their similarities are evident — cyclopamine being 11-deoxojervine and cycloposine being 3-glucosyl-11-deoxojervine.

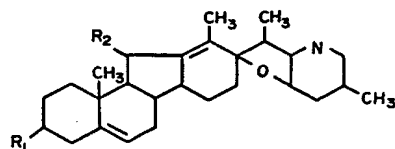
Jervine has been found in many species in the *Veratrum* genus (Kupchan et al., '61). 11-Deoxojervine has been isolated only from the Japanese plant, *V. glandiflorum* (Masamune et al., '64), and *V. californicum* (Keeler, '69b). 3-glucosyl-11-deoxojervine has been isolated only from *V. californicum* (Keeler, '69c).

Kupchan and Suffness ('68) reported on the configuration of jervine. Because of reactions relating jervine to veratramine and 11-deoxojervine (cyclopamine) they conclude that the latter two have the same configuration as jervine. By analogy we can infer that 3-glucosyl-11-deoxojervine is configurationally similar in the steroidal end of the molecule. Thus all share the configuration shown in figure 2 for cyclopamine.

A variety of steroidal compounds (alkaloids, hormones, and sapogenins) have been tested for cyclopic teratogenicity in sheep (Keeler and Binns, '68) (table 1). Errors in assignment of teratogenicity cannot be ruled out, because of the logistically difficult assay in sheep. Animal size, extensive facilities required, and expense precluded testing in large numbers of animals. But assuming errors have not arisen in assignments thus far a rather specific structural requirement is evident.

The three teratogenic compounds differ only in substitution at the 3 and 11 positions and are otherwise structurally and configurationally similar. While the crude assay has not provided a measure of relative activity it can tentatively be concluded that the nature of the substitution at these two positions plays little role in the activity, although substitutions at these positions with substituents inducing lesser polarity (position 3) or greater polarity (position 11) need to be tested.

The closely similar alkaloids veratramine and its glycoside veratrosine, as well as the *O,O,N*-triacetylveratramine derivative (fig. 3), did not produce cyclopia. Veratramine



JERVINE $R_1=OH, R_2=O$
CYCLOPAMINE $R_1=OH, R_2=2H$
CYCLOPOSINE $R_1=GLU, R_2=2H$

Fig. 1 The structures of teratogenic veratrum alkaloids.

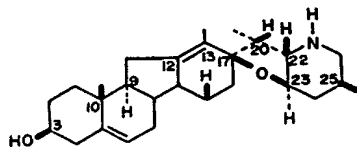
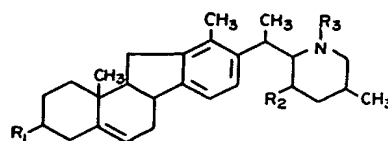


Fig. 2 The configuration of cyclopamine.

TABLE 1
Compounds tested for teratogenicity in sheep

Compounds	Results ¹
Cyclopamine	Active
Cycloposine	Active
Jervine	Active
Rubijervine	Nonactive
Isorubijervine	Nonactive
Veratramine	Nonactive
Pseudojervine	Nonactive
Veratrosine	Nonactive
Protoverine	Nonactive
Cevine	Nonactive
Veracevine	Nonactive
Various mixed veratrum ester alkaloids	Nonactive
Alkaloid Q	Nonactive
3 β ,23, <i>N</i> -triacetylveratramine	Nonactive
1,2-Benzofluorene	Nonactive
Diosgenin	Nonactive
Testosterone	Nonactive
Androstendione	Nonactive
Pregnenolone	Nonactive
Progesterone	Nonactive
Hydrocortisone	Nonactive
Smilagenin	Nonactive

¹ The nonactive compounds produced no cyclopic or related effects. Of the nonactive compounds only veratramine was found to produce any teratogenic effects. The occasional bowing of limbs, joint flexure, and lack of muscular control resulting from veratramine ingestion have been reported (Keeler and Binns, '66b).



VERATRAMINE $R_1=OH, R_2=OH, R_3=H$
TRIACETYLVERATRAMINE $R_1, R_2, R_3=AC$
VERATROSINE $R_1=GLU, R_2=OH, R_3=H$

Fig. 3 The structures of some nonteratogenic alkaloids.

and veratrosine differ structurally and configurationally from cyclopamine and cycloposine only in the lack of oxide ring and presence of complete unsaturation in ring D. Thus one can assume a requirement for either the oxide ring or the resultant partial saturation of ring D or both. While the hydroxyl at position 23 in veratramine is in the β -configuration as is the 17-23 ether bridge in the cyclopamine there would be considerable free rotation about the 17-20 and 22-23 bonds in veratramine. Thus the rigid positioning of the piperidine ring with respect to the steroid system in cyclopamine would be absent in veratramine. Therefore it is tempting to conclude it is the oxide bridge that is important.

Other compounds tested thus far (Keeler and Binns, '68) have not produced cyclopia (table 1). None possessed the fused oxide and piperidine rings (substituted tetrahydrofurylpiperidines) present in the active compounds. The two inactive steroidal saponinins, diosgenin and smilagenin, do possess an oxide ring E, but it is fused to ring D rather than ring F, and ring F is not a piperidine. Both tomatoes and potatoes possess steroidal alkaloids similar to those saponinins and with a terminal piperidine ring F. Pure alkaloids of this type have not yet been tested but both potatoes and tomatoes selected for high total alkaloid content have been fed without effect.

Although substitutions on the steroidal portion of the molecule at positions 3 and 11 did not cause loss of activity it remains to be shown whether other positional substitutions and rearrangement of double bonds on the steroidal portion will alter ac-

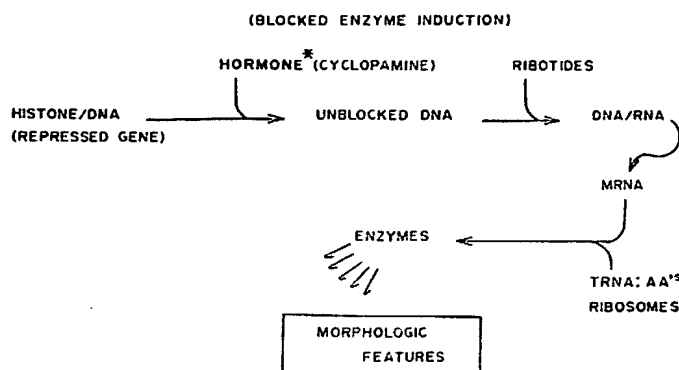


Fig. 4 The proposed mechanism of action of cyclopamine (see text).

tivity. Whether substitution of a steroid ring system at position 17 other than the C-nor-D-homo type or indeed substitution of a nonsteroidal substituent would alter activity is not known.

These questions are important from the point of view of the proposed mechanism of action of these alkaloids (fig. 4). As a working hypothesis it was proposed (Keeler and Binns, '66a) that cyclopamine functions as a competitive inhibitor of an unknown hormone stimulating RNA synthesis. Now that the structure of cyclopamine is known (Keeler, '69b) and more data are available on the role of steroidal hormones in derepression of genetic material (Karlson and Sekeris, '66; Ursprung and Huang, '67; O'Malley, '69) we are even further attracted to the hypothesis. The hypothesis is shown graphically in figure 4 with the competitive inhibition between hormone and cyclopamine occurring at the derepression step to yield unblocked DNA. Whether the mechanism involves: (1) acetylation or phosphorylation of the histone (Allfrey, '69), (2) direct interaction of hormone with histone (Sluyser, '69), (3) other action by hormone, or (4) an effect at the translation level rather than the transcription level (Ursprung and Huang, '67) is peripheral to the basic premise that the teratogenic effect results from the competitive inhibition of hormone and cyclopamine. The premise leads further to the implication that it is the steroidal end of the cyclopamine molecule that allows its function in this general class reaction. Because

of the apparent obligate requirement for the tetrahydrofurylpiperidine end of the molecule it is tempting to propose that this portion confers specificity within this general type reaction.

A number of known reactions of jervine allow preparation of structurally altered compounds whose subsequent testing will answer many questions concerning structural requirements (fig. 5). The 5-6 and 12-13 double bonds of jervine can be rearranged or eliminated readily to determine what role, if any, they play in activity. Reductions of the 12-13 or the 5-6 and 12-13 double bonds can be achieved with a minor product of the latter reduction being a rearrangement isomer with 5-6 and 12-14 unsaturation (Iselin and Wintersteiner, '55). The isomer can be further rearranged (Iselin and Wintersteiner, '55) to yield a compound with 7-8 and 12-14 unsaturation in which the double bonds are in conjugation with the carbonyl at position 11. Acid treatment of jervine (Jacobs and Craig, '44) yields isojervine, which has double bonds at the 5-6, 8-9, and 13-17 positions and in which the ether bridge has been cleaved to leave a hydroxyl at position 23 in the piperidine ring. Comparison of its activity with the nonactivity of veratramine should suggest whether it is the unsaturation of ring D or the lack of ring system rigidity resulting from ether bridge cleavage that causes veratramine to be inactive. Presence or absence of a carbonyl group at position 11 does not alter activity. The carbonyl can be reduced (Iselin and

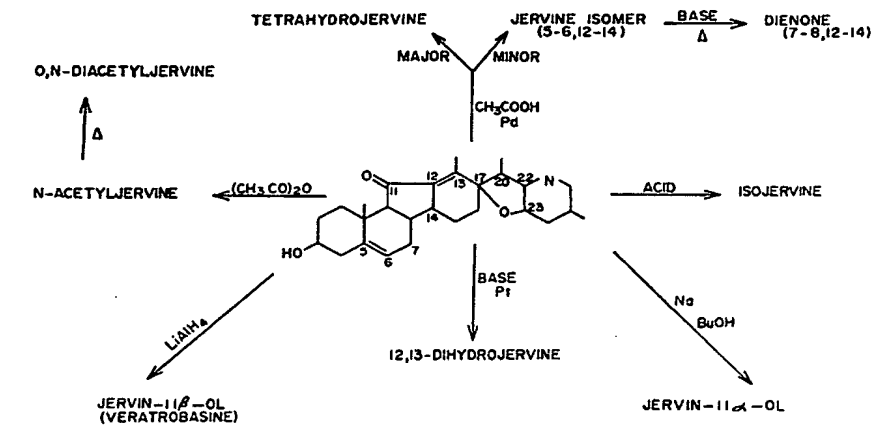


Fig. 5 Preparation of structurally altered jervine derivatives.

Wintersteiner, '56) to yield a 11 β -ol or 11 α -ol product. The greater polarity induced in the molecule by the hydroxyl should help determine whether substituents at that position do indeed play no role in activity. Both the nitrogen on the piperidine and the hydroxyl at position 3 can be blocked by acetylation both in the case of jervine and isojervine (Iselin and Wintersteiner, '55) to test their role in activity. Lastly, one would like to prepare a series of tetrahydrofurylpiperidine derivatives with substituents other than a C-nor-D-homo steroid at position 17 but there are no obvious routes.

The difficulty thus far in studies to determine the precise structural requirements has rested with biological testing rather than with chemical preparations. Active oral doses in sheep have been in the gram range and therefore requirements for derivatives have been overwhelmingly large. Other dosing routes in sheep have not been promising perhaps because of toxicity/dose rate problems.

One would expect dose requirements to decrease by an order of magnitude or more in laboratory animals. However, until recently we had produced no cyclopia in non-ruminant animals, using plant material or purified preparations (Binns and Keeler, '69).

The accompanying report (Keeler, '70a) describes a method that allows production of cyclopia in rabbits using cyclopamine.

This method demonstrates that nonruminants are susceptible and provides us with a manageable assay system for both structural requirement and mechanism studies.

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Application No: 10/682584
Filed: October 9, 2003
For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit C

Kutney JP et al, *Canadian Journal of Chemistry* 1975;53:1796-1817.

Synthetic Studies in the *Veratrum* Alkaloid Series. II.¹ The Total Synthesis of Verarine, Veratramine, Jervine, and Veratrobazine

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Coupling of 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (**1**) with the lithio derivative of 2-ethyl-5-methylpyridine provides the crucial intermediate (**3**) for the subsequent elaboration to verarine. Aromatization of **3** to **4** and reduction of the latter provides a mixture from which *N*-acetyl-5 α ,6-dihydroverarine (**11**) was isolated. Subsequent introduction of the 5,6-double bond in the latter and removal of the *N*-acetate function completed the synthesis of verarine (**31**). In a similar sequence of reactions employing **1** and the lithio derivative of 2-ethyl-3-methoxy-5-methylpyridine, the resultant intermediate (**54**), was elaborated to 5 α ,6-dihydroveratramine (**56**). Due to known conversions of the latter to veratramine (**14**), jervine (**32**), veratrobazine (**33**), and 11-deoxojervine (**34**), the formal total synthesis of these natural products is complete.

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Le couplage de l'acétoxy-3 β (5 α)-étiojervène-12(13) one-17 (**1**) avec le dérivé lithié de l'éthyl-2 méthyl-5 pyridine fournit l'intermédiaire (**3**) crucial pour l'élaboration subséquente de la verarine. L'aromatization de **3** en **4** et la réduction de ce dernier dérivé fournit un mélange duquel on peut isoler la *N*-acétyldihydro-5 α ,6 verarine (**11**). L'introduction subséquente d'une double liaison en position 5,6 et l'élimination de la fonction *N*-acétate, complète la synthèse de la verarine (**31**). Utilisant une séquence semblable de réactions faisant appel à **1** et au dérivé lithié de l'éthyl-2 méthoxy-3 méthyl-5 pyridine, on a obtenu l'intermédiaire (**54**) qui a été transformé en dihydro-5 α ,6 veratramine (**56**). En se basant sur le fait que ce dernier composé peut être transformé, à l'aide de séquences connues, en veratramine (**14**), en jervine (**32**), en veratrobazine (**33**) et en déoxo-11 jervine (**34**), on peut considérer que la synthèse totale formelle de ces produits naturels est maintenant complète.

[Traduit par le journal]

In the previous publication (26) we described the total synthesis of various C-nor-D-homo steroidal analogs to be employed as intermediates in the synthesis of *Veratrum* alkaloids. We also outlined a synthetic plan which envisages the *Veratrum* skeleton as consisting of a C-nor-D-homo steroid or etiojervane portion to which is attached at the 17-position, an appropriate heterocyclic unit. We would now like to present the experiments which demonstrate the application of this plan to the total synthesis of the alkaloid verarine (**31**) (**1**, **2**).

The structure of verarine reveals that the required heterocyclic unit attached to the steroid portion is a 2,5-disubstituted piperidine and it was felt that the latter is readily available from

the appropriate pyridine by well known reduction methods. On this basis, the essential features of the synthetic plan, as outlined in Fig. 1, were to couple the pyridine system at C₁₇ of the already available 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (**1**)¹ and the resultant product **3** could, hopefully by aromatization of ring D (**4**) and subsequent reduction, lead to the desired 5,6-dihydroverarine (**5**, R = R' = H) system. It should be noted here that *both* racemic and optically active forms of **1** are available from total synthesis, the former from our previous work (26, 27) and the latter from that of Sondheimer and co-workers (**3**) who presented a total synthesis of optically active hecogenin from isoandrosterone. For the purposes of the synthetic experiments discussed here we have employed optically active **1** obtained via degradation of hecogenin acetate as outlined in Part I of this series.¹

To evaluate the feasibility of the approach

¹For part I, see ref. 26. For preliminary reports on a portion of this work, see ref. 27.

²Revision received February 4, 1975.

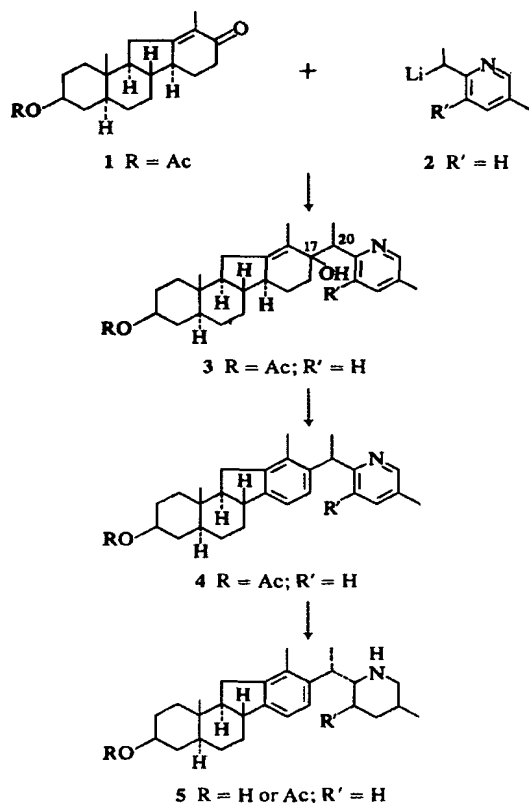
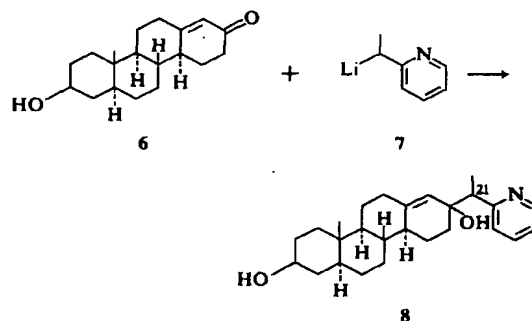


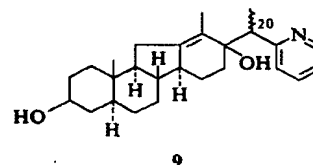
FIG. 1. General outline of synthetic plan for the synthesis of verarine (31).

presented in Fig. 1, the readily available (26, 27) hydrochrysene derivative **6** was utilized as the model substance and this was condensed with the lithio derivative of 2-ethylpyridine (**7**).



Addition of **6** to the red solution of 2-ethylpyridine and methyl lithium provided a reaction mixture which after chromatographic purification yielded the desired product. Its u.v. spectrum showed peaks at 257, 262, and 270 nm corresponding to the pyridine chromophore while the mass spectrum exhibited a peak at m/e 395 corresponding to the parent ion for compound **8**. The absence of a carbonyl peak in the i.r. supported the 1,2-addition of the organolithium derivative to the α,β -unsaturated carbonyl compound as opposed to any 1,4 addition.

This procedure was then extended to the reaction of **7** with 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (**1**). Column chromatography of the products from this reaction gave primarily some material which appeared as one compound on t.l.c. The spectroscopic properties of the reaction product (λ_{max} 257, 262, and 269 nm; m/e 395) were again in accord with the desired coupling product although the n.m.r. spectrum (two doublets in the region τ 8.84) revealed that a mixture of two isomers, most likely epimeric at C₂₀, had been obtained (**9**).



These model studies indicated that the required attachment of a heterocyclic unit at C₁₇ of the C-nor-D-homo compound could be achieved and our efforts turned to the consideration of substituted 2-ethylpyridines which would allow entry to the skeleton of some of the naturally occurring *Veratrum* alkaloids.

Methylation of 2,5-lutidine employing phenyl lithium and methyl iodide afforded the known (4) 2-ethyl-5-methylpyridine and this in the form of its lithium salt (**2**) was condensed with the etiojervene derivative (**1**). The resultant product mixture upon t.l.c. examination, revealed two major components with similar R_f values but attempts to separate these proved fruitless. Conversion to the C₃-acetate derivatives did allow purification of this mixture.

It is to be noted that the coupling reaction creates two new asymmetric centers and consequently there exists the possibility of obtaining

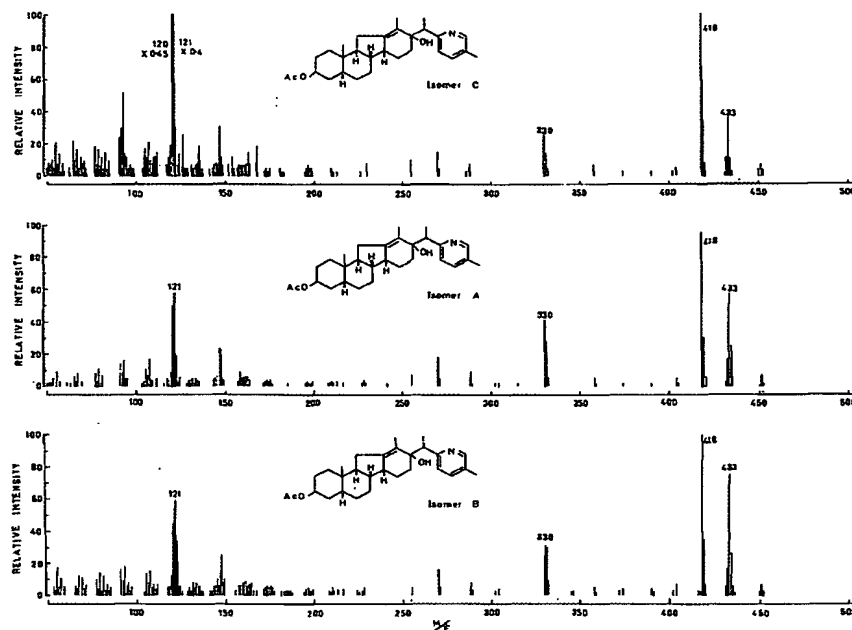


FIG. 2. Mass spectra of isomers A, B, and C.

four compounds possessing the gross structure 3 and differing in stereochemistry at C₁₇ and/or C₂₀. All of these could be obtained in pure form.

One of the major components which could be obtained crystalline, m.p. 189–190°, was designated compound A. Spectral data allowed the assignment of structure 3 to this substance. Apart from the pyridine chromophore in the u.v., the n.m.r. spectrum was particularly instructive. A quartet occurring at τ 6.71 was assigned to the proton at C₂₀ since a decoupling experiment showed this signal to be coupled to the C₂₁ methyl doublet at τ 8.79. The C₁₉ methyl signal appeared as a sharp three-proton singlet at τ 9.21. The pyridine protons were readily discernible in the aromatic region of the spectrum with a one-proton doublet at τ 2.82, $J = 8$ Hz, corresponding to C₂₃H whereas C₂₄H gave rise to two doublets at τ 2.52 ($J = 8$ and 2 Hz) and C₂₅H appeared as a broad singlet at τ 1.67. The mass spectrum of A (Fig. 2) revealed the desired molecular ion (m/e 451) and expected fragmentation pattern.

Column chromatography of the mother liquors from the crystallization of compound A gave a

second crystalline component, m.p. 191–192°, designated as compound B which also revealed spectral data in accord with structure 3. The isomeric nature of B and A is seen from the close similarity in the mass (Fig. 2) and n.m.r. spectra of these compounds. In general the n.m.r. features already noted for A also appear in B although there are some differences in the chemical shifts. The largest differences are seen in the positions of the C₁₈ and C₂₁ methyl group signals and the pyridine protons (see Experimental).

Additional purification of chromatography fractions, obtained during isolation of B by means of preparative layer chromatography, allowed the isolation of two minor components, C and D, which constituted about 4 and 1% of the total product mixture. Neither of these could be induced to crystallize but satisfactory spectral data could also be accumulated for these substances and again the gross structure 3 could be assigned. At this stage of the investigations no assignments of stereochemistry at C₁₇ and/or C₂₁ could be made to these four coupling products but evidence in this direction was obtained during later studies as discussed below.

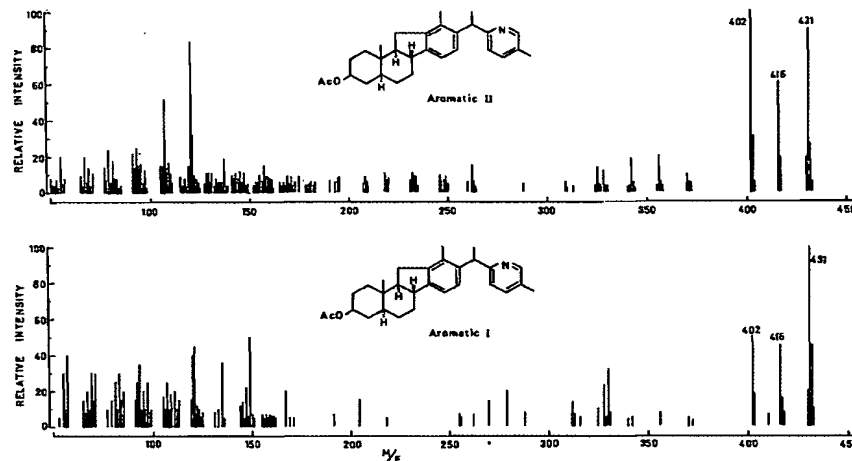


FIG. 3. Mass spectra of aromatic I and II.

The further extension of this sequence to the ring D aromatized series only involved the compounds A and B as the small amounts of C and D which were available precluded any useful reactions.

Compound B was ground with 10% palladized charcoal until the two substances were thoroughly mixed and then the powder was heated at 200° for 10 min. Examination of the reaction product mixture by t.l.c. indicated that compound B had been converted to a new compound possessing a similar R_f value. Separation by preparative layer chromatography indicated this new compound, which was designated 'aromatic II', had been produced in 80% yield. The u.v. spectrum showed no qualitative difference from that of compound B but the extinction coefficient at 270 nm increased from 4130 in the latter to 5400 in this compound. The mass spectrum exhibited a peak at m/e 431 corresponding to the molecular ion for a compound of structure 4. The mass spectrum (Fig. 3) shows a completely different pattern to that observed with the series of compounds A to D. The n.m.r. spectrum was particularly useful in confirming that the desired ring D aromatization had occurred. The most significant feature is the appearance of a second pair of doublets in the downfield region of the spectrum corresponding to the expected AB system for the protons at C_{15} and C_{16} . In addition the broad three-proton singlet which corresponds to the C_{18} methyl in the starting material has disappeared but a new

sharp singlet appears at τ 7.89 in the product in accord with the aromatization of ring D.

Isomer A was similarly treated with 10% Pd/C at 200° and the residue examined by t.l.c. Three compounds could be isolated in pure form by thick-layer chromatography. Two of these could be identified as the α,β -unsaturated ketone (1) and 2-ethyl-5-methylpyridine. The third compound which was formed in 25% yield appeared similar in properties to the aromatic compound from isomer B and was designated 'aromatic I'. The mass spectrum exhibited a peak at m/e 431 corresponding to the molecular ion for a compound of structure 4 and, in general, the fragmentation pattern was very similar to that of aromatic II (Fig. 3). This and other data suggested that these two aromatic substances were merely isomeric at C_{20} and support for this postulate came forth from further experiments as noted below.

In view of the different extent of ring D aromatization encountered with isomers A and B, the relative stability of these two compounds was further investigated. The C_{17} — C_{20} bond cleavage, which occurs with isomer A under the conditions for the D ring aromatization, appears to be very facile since this cleavage also occurs slowly in methanol or ethanol. The cleavage is slightly enhanced when the compound is dissolved in 0.1 *N* methanolic potassium hydroxide. Isomer A is stable in dimethylformamide or benzene but is converted to 3 β -acetoxy-5 α -etiojerv-12(13)-en-

17-one and 2-ethyl-5-methylpyridine when dissolved in aqueous dimethylformamide containing potassium hydroxide (pH 9).

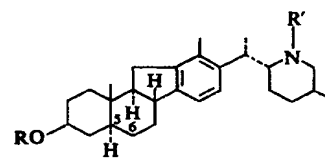
Isomer **B** is stable under the above conditions and shows no C₁₇—C₂₀ bond cleavage. Dehydration of the tertiary alcohol appears to be the main process for both isomers **A** and **B** in acidic methanol solution.

Thus two compounds of structure **4** had been prepared and the selective reduction of the pyridine to a piperidine ring was investigated. One of these aromatic compounds should lead to an isomeric mixture of 5 α ,6-dihydroverarines while the other would lead to a mixture of isomers of similar structure but differing in configuration at C₂₀ from the naturally occurring alkaloid verarine.

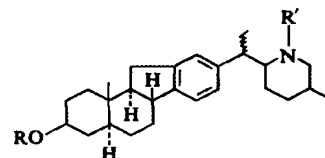
Catalytic reduction (5) of aromatic compound **II** provided a reaction mixture, which upon examination by t.l.c. indicated that four new compounds possessing very similar *R_f* values had been formed. These compounds, which were numbered i to iv in order of decreasing *R_f* values on silica gel, were separated by careful preparative layer chromatography. These compounds were suspected to be isomers of 3-*O*-acetyl-5 α ,6-dihydroverarine (**10**). However, preparation of a sample of this compound from verarine for comparison purposes is difficult since acetylation of verarine yields *N*-acetylverarine or 3-*O*,*N*-diacetylverarine.

None of the four compounds from the hydrogenation of aromatic compound **II** could be induced to crystallize but it was found from other investigations that *N*-acetyl-5 α ,6-dihydroverarine (**11**) crystallizes readily from an ethereal solution. Consequently it was decided to convert the four compounds obtained above to the *N*-acetyl derivatives (gross structure **12**) via acetylation to the 3-*O*,*N*-diacetates (**13**) followed by selective hydrolysis of the 3-acetoxyl group. The conversion to the respective diacetates was accomplished with acetic anhydride–pyridine (1:1) while hydrolysis of the 3-acetoxyl group was achieved with 0.1 *M* potassium hydroxide in methanol.

The appropriate verarine derivatives required for the above comparison were prepared from the more readily available alkaloid veratramine (**14**) essentially according to the scheme developed by Masamune (1) with modifications at appropriate stages (**14** → **15** → **16** → **17**).



10 R = Ac; R' = H
11 R = H; R' = Ac



12 R = H; R' = Ac
13 R = R' = Ac

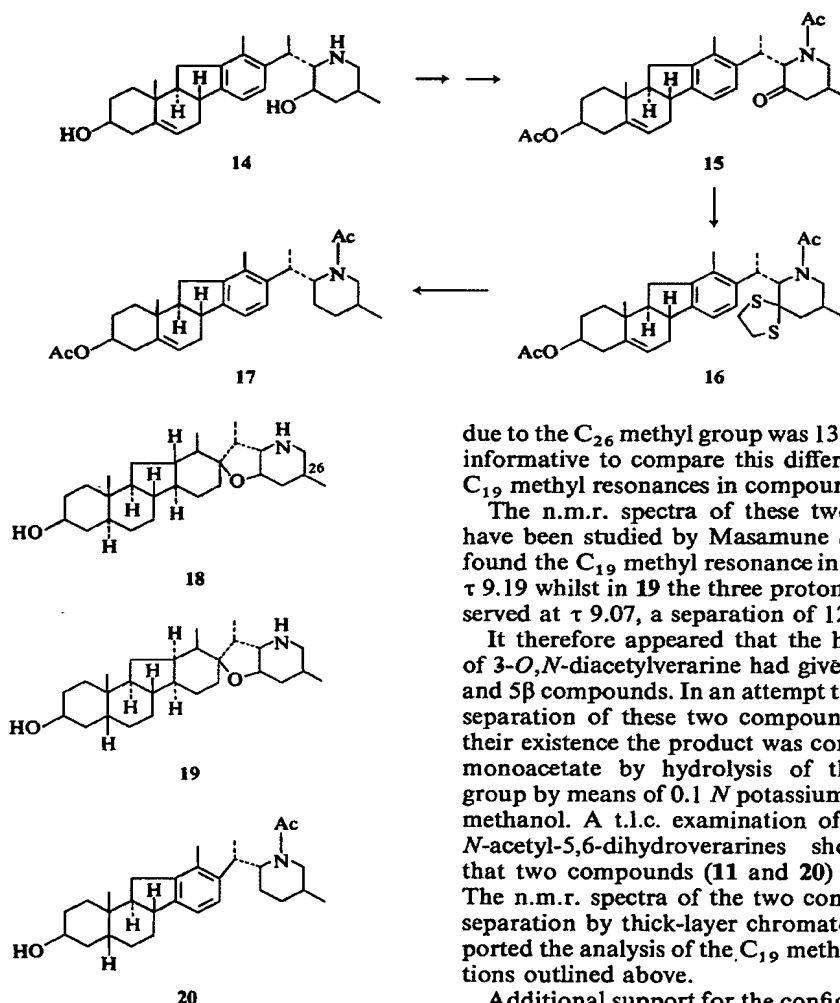
The diacetate **17** was identified by t.l.c. comparison with an authentic sample³, melting point, rotation, mass spectrum, and n.m.r., all of which were in accord with the published data (6). Subsequent hydrolysis of the 3-acetoxyl compound gave *N*-acetylverarine.

Hydrogenation of the 5,6-double bond in veratramine employing Adams' catalyst in acetic acid had been reported to give mainly the 5 α ,6-dihydroveratramine (7). By analogy it was expected that the hydrogenation of the 5,6-double bond in 3-*O*,*N*-diacetylverarine (**17**) using these conditions would provide mainly the desired 3-*O*,*N*-diacetyl-5 α ,6-dihydroverarine. This hydrogenation was carried out and careful examination of the product by t.l.c. indicated that only one compound had been formed. However, a close examination of the n.m.r. spectrum particularly in the region τ 8.5–9.5 indicated that both the 5 α and the 5 β compounds had been formed.

The n.m.r. spectrum of 3-*O*,*N*-diacetylverarine exhibited a three-proton doublet at τ 9.02 which was attributed to the C₂₆ methyl while the C₁₉ methyl appeared as a three-proton singlet at 8.85.

The n.m.r. spectrum of the hydrogenation product exhibited a sharp singlet at 8.92 and a doublet at 9.03. The upfield half of the doublet centered at 9.03 was, however, very intense and the integral of this doublet corresponded to 4.5 protons. From the excellent n.m.r. study of 22,27-

³We are grateful to Dr. J. Tomko, Slovak Academy of Sciences, Bratislava, Czechoslovakia, for a gift of this sample.



imino-17,23-oxidojervane derivatives by Masamune *et al.* (8) one would expect the C_{19} methyl signal for the 5α compound to be at a higher field position than in the 5β compound. This difference is due to the shielding of the C_{19} methyl group by the ring A protons which are in close proximity in the 5α derivatives but removed to a large extent in the 5β derivatives. This effect has been noted in various steroids (9) where the stereochemistry of the molecule is fixed.

The difference in chemical shift between the singlet at 8.92 and the upfield half of the doublet

due to the C_{26} methyl group was 13 Hz and it was informative to compare this difference with the C_{19} methyl resonances in compounds 18 and 19.

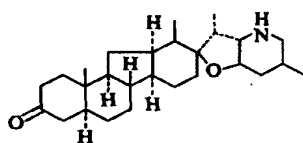
The n.m.r. spectra of these two compounds have been studied by Masamune *et al.* (8) who found the C_{19} methyl resonance in 18 to occur at τ 9.19 whilst in 19 the three proton-singlet is observed at τ 9.07, a separation of 12 Hz.

It therefore appeared that the hydrogenation of 3-*O,N*-diacetylverarine had given both the 5α and 5β compounds. In an attempt to obtain some separation of these two compounds and verify their existence the product was converted to the monoacetate by hydrolysis of the 3-acetoxy group by means of 0.1 *N* potassium hydroxide in methanol. A t.l.c. examination of the resulting *N*-acetyl-5,6-dihydroverarines showed clearly that two compounds (11 and 20) were present. The n.m.r. spectra of the two compounds after separation by thick-layer chromatography, supported the analysis of the C_{19} methyl group positions outlined above.

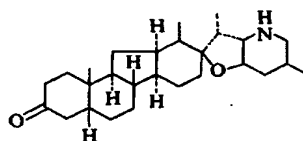
Additional support for the configurational differences at C_5 was evident from the C_3 proton signals (τ 5.97, narrow multiplet in 11, equatorial H; 6.3–6.5, broad multiplet in 20, axial H).

It was vital to provide additional data which establish more firmly the identity and stereochemistry of these compounds. The o.r.d. curves of 3-keto steroids with the 5α and 5β configuration have been extensively studied and Masamune *et al.* (8) have recorded the o.r.d. curves of compounds 21 and 22. These latter substances provide a very good analogy with the compounds under consideration.

Compound 21 exhibits a strong positive Cotton



21

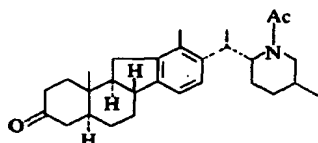


22

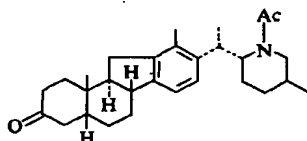
effect curve with values of $[\phi]_{265}^{\text{trough}} - 1180^\circ$ whereas 22 exhibits a negative Cotton effect curve with values of $[\phi]_{304}^{\text{peak}} + 2740^\circ$ and $[\phi]_{265}^{\text{trough}} - 1180^\circ$ whereas 22 exhibits a negative Cotton effect curve with values of $[\phi]_{304}^{\text{trough}} + 265^\circ$ and $[\phi]_{265}^{\text{peak}} + 2340^\circ$.

The hydrogenation products which had tentatively been assigned the 5 α (11) and 5 β (20) configurations were converted to the respective 3-keto compounds (23 and 24) using Jones' reagent as the oxidizing agent. The presence of the *N*-acetyl group tended to complicate the o.r.d. curves to some extent since it had a strong absorption at 230 nm but the absorption at 300 nm was not sufficient to affect the curve in this region. Compound 23 showed a strong positive Cotton effect curve with a peak at 305 nm while compound 24 showed only a very weak trough at 305 nm.

After this work had been completed, a publication (10) appeared in which the hydrogenation of veratramine employing Adams' catalyst in acetic acid has been more closely examined. Saito (7) had reported only the formation of the 5 α -6,



23



24



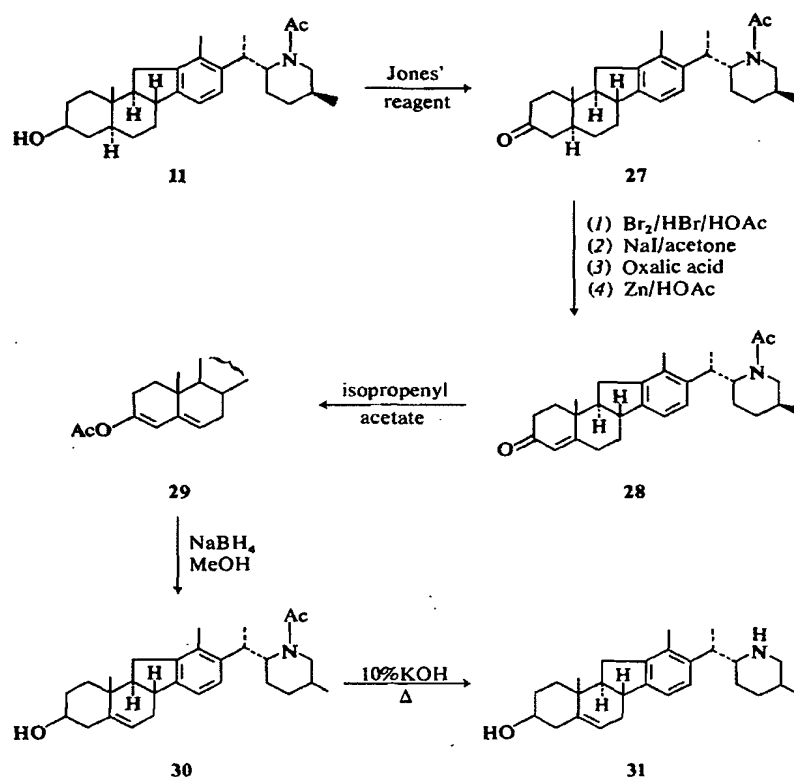
25 R = H
26 R = Ac

dihydroveratramine but the recent work indicates that a mixture of 5 α ,6-dihydroveratramine (41%) and 5 β ,6-dihydroveratramine (44%) is produced. It is interesting to note that these workers also distinguished the two compounds on the basis of the chemical shifts of the C₁₉ methyl resonances in the n.m.r. spectra.

Having established the identity of the desired *N*-acetyl-5 α ,6-dihydroveratramine (11) the comparison of this compound with the *N*-acetyl derivatives of the four compounds obtained from the hydrogenation of aromatic compound 2 was now undertaken. These crystalline acetates of compounds i-iv were compared in the usual manner (m.p., t.l.c., i.r., n.m.r., and mass spectra) with authentic 11. It was established that i, *N*-acetate, was identical in every respect with 11 as prepared above.

Careful analysis of the mass spectra of all five compounds (11 and i-iv) reveals an essentially identical fragmentation pattern with very intense peaks at *m/e* 140 and 98. Budzikiewicz (11) has examined the mass spectra of veratramine, *N*-acetylveratramine, and verarine and has postulated cleavage of the benzylic C₂₀—C₂₂ bond which, in the present instance, would allow the fragments 25 and 26 to account for strong peaks at *m/e* 98 and 141, respectively.

Aromatic compound I which was obtained in 25% yield from isomer A was subjected to hydrogenation under the same conditions as applied to aromatic compound II. Examination of the product by t.l.c. indicated that four new compounds of similar *R_f* values had been formed. For the sake of convenience these were designated v-viii in order of decreasing *R_f* values on a thin-layer chromatoplate. These compounds were separated by thick-layer chromatography and then converted via the diacetates to the respective *N*-acetyl derivatives. A t.l.c. comparison of the four *N*-acetates with *N*-acetyl-5 α ,6-dihydroveratramine (11) indicated that only compounds v and vi had a similar *R_f* value. Surprisingly none of these *N*-acetyl derivatives could be induced to crystallize from ether in contrast to *N*-acetyl-5 α ,6-dihy-

FIG. 4. Conversion of *N*-acetyl-5 α ,6-dihydroverarine (11) to verarine (31).

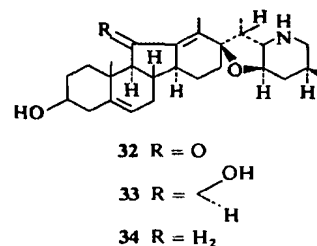
droverarine and the *N*-acetates of compounds i-iv which all crystallize very readily from this solvent. The mass spectra of the compounds v-viii exhibited a peak at *m/e* 437 corresponding to the molecular ion for *N*-acetyl-5 α ,6-dihydroverarine as well as prominent peaks at *m/e* 98 and 140.

To complete the synthesis of verarine (31) it was necessary to introduce the 5,6-double bond and remove the *N*-acetate function in *N*-acetyl-5 α ,6-dihydroverarine (11). The steps involved in achieving these goals are outlined in Fig. 4. The procedure for introduction of the double bond is taken from the investigations of Evans *et al.* (12) in the cortisone series. A similar pathway has been employed by Johnson *et al.* (13) in the conversion of 23-*O,N*-dibenzoyl-5 α ,6-dihydroveratramine to veratramine.

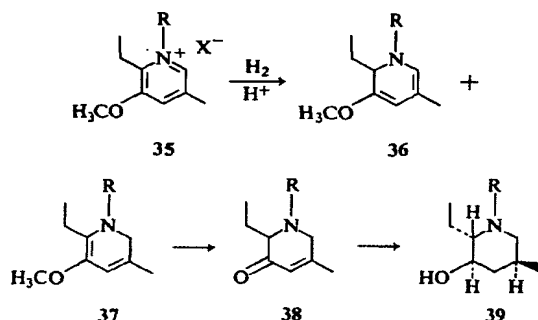
Having completed the synthesis of the simplest member of the *Veratrum* series, it was decided to extend the above strategy to encompass some of

the more oxygenated *Veratrum* alkaloids and 5 α ,6-dihydroveratramine (56) became the target compound. Since the latter had already been converted to veratramine (14) (13), jervine (32) (14, 15), veratrobazine (33) (15, 16), and 11-deoxojervine (34) (17), a synthesis of this intermediate represents in a formal sense the total synthesis of these natural products.

Analysis of the structure of veratramine, for



example, reveals that the substituents attached to the three chiral centers (C_{22} , C_{23} , and C_{25}) involving the piperidine ring are in equatorial orientations. It was thus felt that reduction of an appropriately substituted pyridinium system, under equilibrating conditions, should lead predominantly to the desired stereochemistry in the resultant product (see $35 \rightarrow 36 + 37 \rightarrow 38 \rightarrow 39$).



The obvious heterocyclic unit required for achieving the synthesis in this direction was an unknown pyridine derivative, 2-ethyl-3-hydroxy-5-methylpyridine, and its synthesis was first considered. We chose an approach which made use of the reaction of furyl ketones with ammonia (18) since it seemed to be more direct and suitable for our purposes. In this connection we have developed two independent sequences for the synthesis of the desired pyridine. In effect, both sequences depend on the availability of crucial

furan intermediates and therefore the synthesis of these substances was initially considered. Figures 5 and 6 outline the reactions involved in the preparation of these materials. Figure 5 reveals the preparation of 2-propionyl-4-methylfuran (44) via a Friedel-Crafts reaction on 3-methylfuran.

A survey of the literature at this time indicated that there had been no work on the Friedel-Crafts reaction of 3-methylfuran. In fact only limited data on the electrophilic substitution of 3-alkylfurans in general was available and the data indicated that the substitution takes place exclusively at the 2-position (19-21). However, we did not rule out the possibility of substitution at the 5-position and hence decided to investigate this further. The desired starting material, 3-methylfuran (42), was prepared according to the procedure of Cornforth (22, 23) as outlined in Fig. 5.

Reaction of 3-methylfuran with propionic anhydride and orthophosphoric acid as the catalyst at 60° provided a product which upon examination by v.p.c. indicated that it was a mixture of two compounds in the ratio 70:30. Separation of the mixture by means of preparative v.p.c. using a FFAP column allowed the isolation of 2-propionyl-3-methylfuran (43) and 2-propionyl-4-methylfuran (44).

It is noteworthy that indeed substitution takes place at both C_2 and C_5 positions of 3-methylfuran and this was the first demonstrated example of electrophilic substitution at the C_5 position of

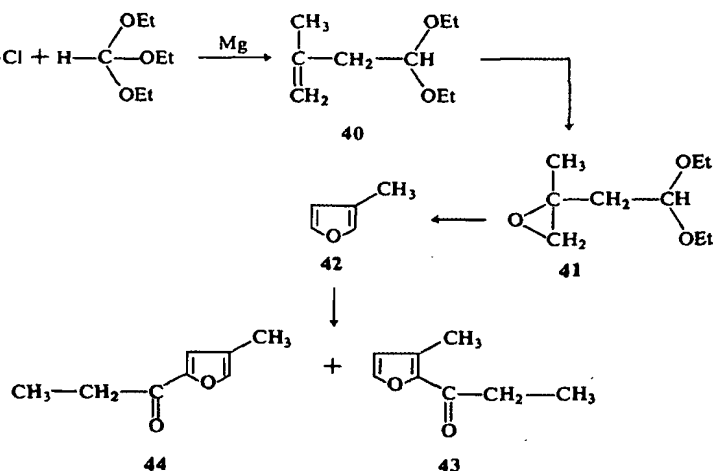


FIG. 5. Synthesis and propionylation of 3-methylfuran.

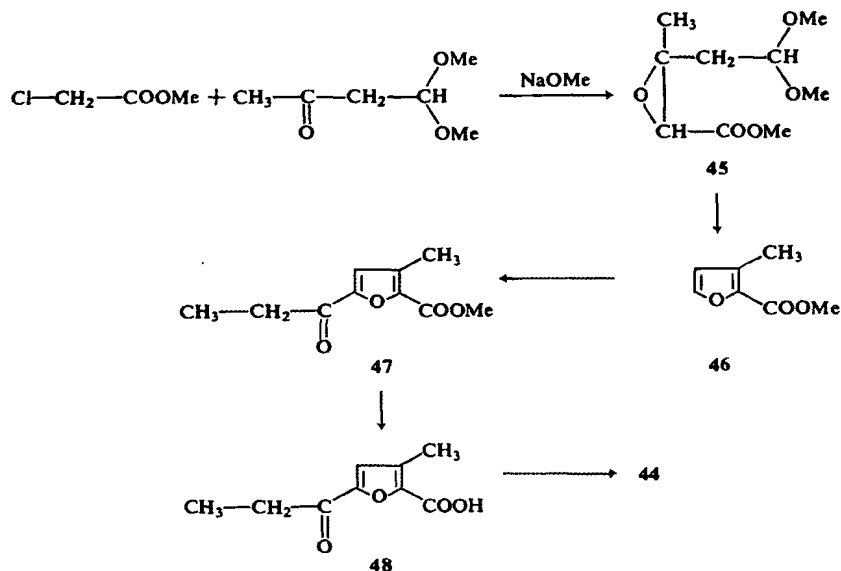


FIG. 6. Synthesis of 2-propionyl-4-methylfuran (44).

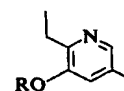
a 3-alkylfuran unsubstituted at the 2-position. Documented data indicate that only furans with electron-withdrawing substituents (*e.g.*, —COOR group) at C₃ can direct substitution to C₅, whereas the presence of electron-donating substituents invariably direct the substitution to C₂ (20). A more detailed investigation is now reported from our laboratory (24).

The difficulties encountered in the separation of the reaction mixture as well as the poor yield of the desired ketone **44** compelled us to discard the above sequence in favor of an alternate direct route to the preparation of this compound.

This latter route makes use of methyl 3-methyl-2-furoate (**46**) as the starting material. The preparation of this compound by a known procedure (25) and the subsequent reactions leading to 2-propionyl-4-methylfuran are schematically presented in Fig. 6.

The next stage in the synthesis was the conversion of 2-propionyl-4-methylfuran to 2-ethyl-3-hydroxy-5-methylpyridine (**49**). This conversion was effected by a known procedure (18). The resultant product (**49**) was crystalline and revealed spectral data in agreement with the assigned structure (λ_{max} 287 nm; τ 8.75, triplet, CH₃CH₂; 7.8, singlet, C₅—CH₃; 2.9 and 2.1, singlets, C₄ and C₆H).

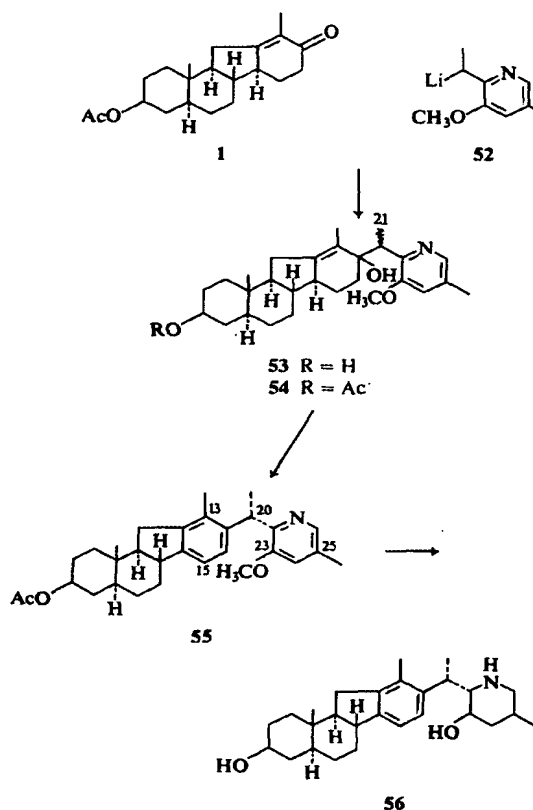
In our initial studies on the coupling of the substituted pyridine with the steroidal enone, it was decided to use the lithio derivative of 2-ethyl-3-*O*-benzyl-5-methylpyridine (**50**). This compound was prepared by heating a solution of 2-ethyl-3-hydroxy-5-methylpyridine in aqueous



- 49** R = H
50 R = CH₂C₆H₅
51 R = CH₃

sodium hydroxide with benzyl chloride. However these investigations were unsuccessful and hence we discarded the use of the *O*-benzyl ether in favor of the corresponding *O*-methyl ether (**51**) which was readily available from the reaction of **49** with diazomethane.

Thus with the desired steroidal enone, 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (**1**), and the appropriately substituted pyridine, 2-ethyl-3-methoxy-5-methylpyridine (**51**), in hand the stage was set for the coupling reaction of these units. The actual reaction sequence employed for the synthesis of 5 α ,6-dihydroveratramine (**56**) is

FIG. 7. Synthesis of 5 α ,6-dihydroveratramine (56).

shown in Fig. 7 and follows the strategy outlined previously.

Coupling of the lithio derivative 52 and 1 provided a reaction product which in the u.v. spectrum (λ_{max} 283 nm) showed a pyridine chromophore while the mass spectrum exhibited a peak at m/e 439 corresponding to the parent ion for a substance bearing the gross structure 53.

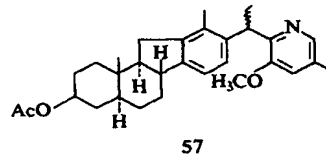
That the material obtained above, although homogeneous on t.l.c., was a mixture of two compounds was indicated by the n.m.r. spectrum which exhibited two sharp singlets at τ 6.21 and 6.24 due to the presence of two methoxyl groups. Two doublets were also seen at τ 8.81 and 8.84 which were probably due to the C_{21} methyl group in each of the two compounds. Attempts at the separation of this mixture by chromatographic techniques were unsuccessful

since both the compounds had identical R_f values on t.l.c. in numerous solvent systems.

In an attempt to achieve separation of these two compounds the mixture was subjected to acetylation by means of acetic anhydride and pyridine. The resulting reaction mixture on t.l.c. examination showed the presence of two compounds possessing very different R_f values. These were separated by column chromatography on alumina. For the sake of discussion the more polar compound is referred to as compound E while the less polar one is designated as compound F.

Examination of compound E revealed the following spectral data. The u.v. spectrum showed a peak of 283 nm with a shoulder at 225 nm indicative of the pyridine chromophore while the mass spectrum exhibited the parent peak at m/e 463 which indicated that dehydration of the coupled product (54) has occurred in the mass spectrometer. The n.m.r. spectrum of E was particularly instructive and a detailed analysis was carried out. Thus a sharp singlet at τ 9.22 with a three-proton integral was assigned to the C_{19} methyl resonance. The doublet at 8.82 was attributed to the C_{21} methyl protons, which were coupled with the C_{20} proton, $J_{20,21} = 7.0$ Hz. The sharp singlets at τ 8.79, 8.0, and 7.7 were due to C_{18} methyl, C_3 acetate, and C_{26} methyl protons, respectively, while the three proton signal at 6.22 was easily recognized as due to the C_{23} methoxyl protons. The protons on the pyridine ring appeared as sharp singlets at τ 3.08 ($C_{24}H$) and 2.0 ($C_{27}H$).

The u.v. spectrum of compound F was similar to that of E while the mass spectrum exhibited the parent peak at m/e 461 corresponding to an ion which accounts for the loss of 20 units from the desired compound of gross structure 54. In the n.m.r. spectrum, the C_{18} methyl signal was shifted downfield (τ 7.7) and was in the position expected for a methyl group on an aromatic ring. Further evidence in support of aromatization of ring D came from an examination of the low field (τ 4–2) region in the spectrum where, in



addition to the pyridine proton signals, an AB quartet centered at τ 3.05 was noted. This signal is clearly due to the C_{15} and C_{16} protons in a ring D aromatic compound with the expected large *ortho* coupling ($J = 7$ Hz).

Since the above spectral data was consistent for the structure 57, it was clear that compound F arises via dehydration of the C_{17} hydroxyl function in the condensation product (53) followed by aromatization of the resulting diene.

It can be seen from Fig. 7 that the coupling reaction generates two chiral centers and consequently there exists the possibility of obtaining four compounds possessing structure 53. However in our studies only two compounds could be isolated and characterized.

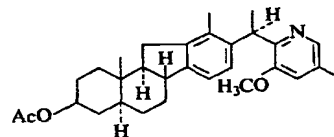
The above results suggest that the coupling reaction afforded a mixture of alcohols of gross structure 53 and that one of them fortuitously underwent dehydration followed by aromatization during the subsequent work-up. There existed the possibility of this sequence of reactions occurring either during acetylation of the reaction mixture or during the chromatographic separation on alumina.

We set out to explore this phenomenon by monitoring a typical experiment by n.m.r. spectroscopy. The product obtained in the coupling reaction was subjected directly to acetylation with acetic anhydride and pyridine at room temperature. After allowing the reaction mixture to stand overnight followed by the usual work-up, the n.m.r. spectrum of this material displayed the low field AB quartet noted earlier. The results of the experiment allowed us to conclude that one of the two alcohols undergoes dehydration and aromatization to F (57) during the acetylation step.

In our subsequent studies we have been able to convert the alcohol acetate 54 (compound E) to an aromatic compound isomeric with F. In a typical experiment, compound E was reacted with palladized charcoal at 200° and three compounds, the α,β -unsaturated ketone 1, 2-ethyl-3-methoxy-5-methoxypyridine (52), and a new substance designated as compound G, were isolated in a pure state. The spectral properties of G were remarkably similar to those of F and it was clear that these compounds were diastereoisomeric at C_{20} , the chiral center created during the coupling reaction.

Since in subsequent experiments the aromatic

compound F gave rise to compounds in the natural series, the stereochemistry at C_{20} in this compound is known and is indicated in 55. This leads to the conclusion that compound G is the C_{20} epimer of F and has the stereochemistry as indicated in 58.



58

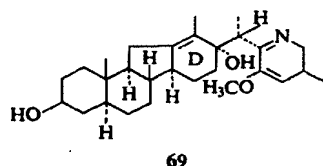
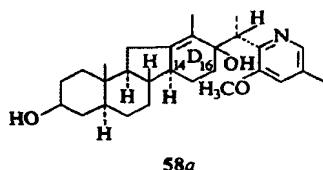
After having established that the condensation product of 2-ethyl-3-methoxy-5-methylpyridine and 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one is a mixture of epimeric alcohols 53 and that one of these alcohols undergoes dehydration and aromatization to give compound F during the subsequent work-up, we set out to assign the stereochemistry of the condensation products. One of the several rational interpretations of the results is advanced in the following paragraphs. We wish to emphasize that we are unable at this time to distinguish clearly between several alternative explanations.

It is clear that several important factors must be considered in proposing any stereochemical assignments to the condensation products possessing the gross structure 53. (i) Is the stereochemistry at C_{20} determined during the approach of the anion to form the C_{17} — C_{20} bond or is the product composition a result of equilibration after the initial reaction has occurred? It is well known that protons adjacent to a pyridine ring (as at C_{20}) are readily removed by strong base. (ii) Is the approach of the anion equally favorable from the 'equatorial' and 'axial' sides of the molecule since this factor determines the stereochemistry at C_{17} ? With regard to the latter, an investigation of molecular models quickly revealed that no distinct preference for either approach is apparent. In other words, one would expect the epimers at C_{17} as the most likely course of events. In the explanation given below we have assumed that such is the case, i.e., the two condensation products differ in stereochemistry at C_{17} but not at C_{20} . In actual fact the discussion below indicates that the results obtained are explicable, regardless whether a definite stereochemistry is initially assigned to C_{20} in the condensation products. For the sake of clarity the

particular stereochemistry chosen for C₂₀ was the one which is present in the natural *Veratrum* series. The reasons for the latter choice are two-fold: (a) there is a minimal interaction between the various groups on ring D and the heterocyclic portion when this stereochemistry is considered and (b) one of the above condensation products leads to 5 α ,6-dihydroveratramine.

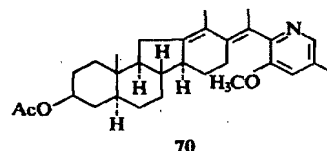
Once a tentative assignment of the stereochemistry at C₂₀ in the condensation products is advanced one is left with the task of advancing a reasonable explanation for the difference in the stability of the alcohols and the observed facile dehydration followed by aromatization in one case as opposed to the sluggishness in the other. An acceptable explanation for the difference in their behaviour is given in the following paragraphs. Here again, it should be emphasized that there can be alternate explanations but the one chosen appears to be the most reasonable.

If we consider the 'axial' alcohol (58a) and its possible conversion to the ring D aromatic derivative (55) it is clear that the allylic hydroxyl can undergo 1,4-elimination making use of the C₁₄ proton, or alternatively a 1,2-elimination involving the α -(axial) proton at C₁₆ is also a feasible process. Either of these eliminations would yield a highly strained cyclohexadiene system (with regard to ring D) which would be expected to aromatize rapidly to yield a compound of structure 55.



On the other hand, examination of molecular models for the 'equatorial' alcohol (69) reveals that in this compound there are no ring D protons which are *trans* and coplanar to the hydroxyl group. The desired stereochemistry for the elimination can be attained at C₂₀ by rotation around the C₁₇—C₂₀ bond but such a process

leads to an eclipsing of the C₁₈ and C₂₁ methyl groups and an increase in nonbonded interactions between ring D (particularly C₁₆) and the substituents on the heterocyclic ring. Hence the sluggishness of this alcohol to dehydrate may be related to the energy required to force the molecule into this sterically unfavorable conformation. The higher temperature (200°) as noted above, under which this alcohol converts to a substance possessing an aromatic D ring would then provide the necessary energy. Dehydration in this event will lead to a diene 70, which via



double bond rearrangement and aromatization would provide the aromatic compound 57. Obviously such a process can lead to epimerization at C₂₀. In fact the aromatic compound (G) isolated in this reaction is isomeric with compound F and must therefore be its C₂₀ epimer (*i.e.* 58).

Now that the desired D ring aromatized compound had been obtained, it was essential to find suitable methods to reduce the pyridine ring to the appropriately functionalized piperidine moiety present in veratramine. For this purpose catalytic hydrogenation was selected as the method of choice. Compound F when subjected to hydrogenation in ethanol at pressures ranging from 40–65 p.s.i. did not undergo any change. However, it was found that this substance undergoes hydrogenation in an acid-alcohol medium (Pt as catalyst). Thin-layer chromatography examination of the reaction product revealed the presence of three major compounds along with trace amounts of several other components. One of the major compounds had the same R_f value as 5 α ,6-dihydroveratramine. Indeed, separation of the mixture afforded an 18% yield of 5 α ,6-dihydroveratramine (56), identical with the natural sample prepared by the hydrogenation of veratramine (7, 10) (*i.e.*, n.m.r., mass spectra, mixture m.p.).

The two other compounds isolated in the hydrogenation experiment were found to be extremely unstable and the limited quantities of these available prevented any meaningful investigation. However, a preliminary inspection of the n.m.r. spectra of the impure compounds

revealed that in both instances the pyridine ring was only partially reduced (to the dihydro or tetrahydropyridine stage) while the methoxyl group was still intact.

As mentioned earlier, the synthesis of 5 α ,6-dihydroveratramine was sufficient for our purpose because this material has already been converted to veratramine (14), jervine (32), veratrobazine (33), and 11-deoxojervine (34).

Experimental

All details concerning spectral measurements, chromatographic separations, etc., are described in part I of this series.

18-Nor-C-homo-26-nor-22,27-iminojerva-12 α (13), 22,24,27-tetraene-3 β ,17-diol (8)

An ether solution of 1.88 M methyl lithium (1.0 ml) was added to anhydrous tetrahydrofuran (4 ml) in a round-bottom flask which had been flame dried and flushed with dry nitrogen. A tetrahydrofuran solution of 2.0 M 2-ethylpyridine (1 ml) was added immediately and the mixture refluxed under nitrogen for 30 min. During this time the solution developed a deep red color. After the period of reflux the α,β -unsaturated ketone (6) in the form of a fine powder was added slowly until the color faded (120 mg) and the reaction mixture refluxed for a further 30 min while the flask was still under nitrogen. Water was then added cautiously to destroy any alkyl lithium remaining and the resulting mixture diluted with ether (10 ml). The organic phase was separated and washed with water (4 $\times$ 10 ml), before drying over anhydrous sodium sulfate. Evaporation of the ether gave a light oil (100 mg) which appeared as one major and several minor components when examined by thin-layer chromatography. The major component was obtained pure by preparative thin-layer chromatography on Woelm neutral alumina (20 $\times$ 20 cm, 0.4 mm, 2% methanol in chloroform). Spectral data indicated this was the desired condensation product (8) although the n.m.r. indicates the material is probably a mixture of two isomers; i.r.: 6.3, 6.4 (pyridine), 3.1 μ (hydroxyl); u.v.: $\lambda_{\max}$: 257 (3.55), 262 (3.58), 270 nm (3.45); n.m.r.: 9.26 (angular methyl, 3H, singlet), 8.8, 8.75 (two overlapping doublets due to the presence of two isomers in the product ($\text{CH}_3\text{—C—H}$, $J=7$ Hz, 3H), 6.53 (CH_3CH , 1H, $J=7$ Hz, quartet), 5.1 (C_{13} , 1H, singlet), 2.0, 3.0 (pyridine, 3H, multiplet), 1.5 (pyridine, 1H, multiplet).

26-Nor-22,27-iminojerva-12 (13), 22,24,27-tetraene-3 β ,17-diol (9)

An ether solution of 1.88 M methyl lithium (1.0 ml) was added to anhydrous tetrahydrofuran in a dry round-bottom flask under nitrogen. A solution of 2-ethylpyridine (1.0 ml of 2.0 M) in tetrahydrofuran was added immediately and the mixture refluxed for 30 min during which time a deep red color developed. Addition of 3 β -acetoxy 5 α -etiojerv-12(13)-en-17-one (1) in the form of a finely ground dry solid was continued until the color faded (90 mg added) and the mixture stirred for a further 30 min, while the flask was still under nitrogen. Water was added to quench the reaction and the mixture diluted with ether (10 ml). The organic phase was separated and then washed

with water (4 $\times$ 10 ml) before drying over anhydrous sodium sulfate. Examination of the ethereal extract by thin-layer chromatography indicated one major component which appeared as a bright yellow spot when the chromatoplate was developed with 1:1 antimony pentachloride-carbon tetrachloride (1:1) spray reagent. Column chromatography on alumina (Shawinigan, activity III, 25 g) eluting with benzene-chloroform (4:1) enabled purification of this component which was crystallized from benzene-ether as needles (42 mg), m.p. 204–206°; i.r. (CHCl_3): 6.30, 6.41 μ (pyridine); u.v.: $\lambda_{\max}$ 257sh, 262.5 (3.58), 269sh nm; n.m.r.: 9.26 (angular methyl, 3H, singlet), 8.84 (C_{21} methyl, 3H, $J_{20,21}=7$ Hz, doublet), 8.26 (C_{18} methyl, 3H, singlet), 2.8 (pyridine, 2H, multiplet), 2.34 (pyridine, 1H, multiplet), 1.5 (pyridine, 1H, multiplet) mass spectrometry: m/e 395 (M^+), prominent peaks at m/e 377, 362, 288.

Anal. Calcd. for $\text{C}_{26}\text{H}_{35}\text{O}_2\text{N}$: C, 78.94; H, 9.43; N, 3.54. Found: C, 78.85; H, 9.50; N, 3.50.

2-Ethyl-5-methylpyridine

An anhydrous ether solution (250 ml) of bromobenzene (31.4 g, 0.2 mol) was added slowly to a dry round-bottom flask which had been flushed with nitrogen and which contained lithium wire (2.8 g, 0.4 mol). When the lithium had largely dissolved an ethereal solution (100 ml) of 2,5-lutidine (21 g, 0.2 mol) was added and the mixture refluxed for 30 min, during which time a deep red color developed in the solution. Methyl iodide (14.2 g, 0.1 mol) in ether (40 ml) was added slowly to this red solution over a period of 15 min and the mixture stirred for 10 min. The excess lithium and phenyl lithium were removed by the careful addition of water (100 ml) to the reaction mixture while the flask was still under nitrogen. A further 200 ml of water was then added and the organic phase separated, and washed with water (2 $\times$ 50 ml). The ethereal solution was reduced in volume to 40 ml by use of a rotary evaporator. This 40 ml was then carefully distilled at a pressure of 10 mm, employing a spinning band column. The first fraction, distilling at 50–55° was shown by n.m.r. to be recovered 2,5-lutidine whereas that distilling at 62–63° was shown to be 2-ethyl-5-methylpyridine; i.r. (film): 6.3, 6.4, 6.8, 7.02 μ ; u.v.: $\lambda_{\max}$ 265sh (3.54), 269 (3.60), 276 nm (3.48); n.m.r.: 8.70 (CH_3CH_2 , 3H, $J=7.5$ Hz, triplet), 7.72 ($\text{CH}_3\text{—C}$, 3H, singlet), 7.20 (CH_2CH_3 , 2H, $J=7.5$ Hz, quartet), 2.93 (C_5 , 1H, $J_{3,4}=8$ Hz, doublet), 2.59 (C_4 , 1H, $J_{4,3}=8.0$ and $J_{4,6}=2.0$ Hz, two doublets), 1.63 (C_6 , 1H, $J_{6,4}=2.0$ Hz, doublet). Picrate derivative from acetone, m.p. 143–144° (lit. (7) m.p. 144°).

3 β -Acetoxy-22,27-iminojerva-12 (13), 22,24,27-tetraene-17-ol (3)

A 2.05 M ethereal solution of methyl lithium (25 ml, 0.051 mol) was added to a flame dried round-bottom flask containing anhydrous tetrahydrofuran (40 ml) under nitrogen. A mixture of anhydrous tetrahydrofuran (10 ml) and 2-ethyl-5-methylpyridine (6 g, 0.05 mol) was added immediately and the reaction mixture refluxed for 1½ h. During this period a deep red color developed in the solution. A faint pink color persisted after 3 β -acetoxy-5 α -etiojerv-12-en-17-one (1) (2.0 g, 0.006 mol) was added as a fine powder to the cooled solution and the mixture stirred for 10 min. Water (50 ml) was added cautiously to the reaction mixture while still under nitrogen, followed by ether (50 ml) prior to separation of the organic and

aqueous phases. The organic phase was washed with water (3×25 ml) and then dried over anhydrous sodium sulfate. Removal of the solvent *in vacuo* gave a light oil (2.3 g) which appeared to consist of two new components when examined by thin-layer chromatography. Attempts to obtain these compounds in a pure state were unsuccessful. However a small scale investigation indicated that the compounds could be separated more readily as the C_3 acetates rather than as the C_3 alcohols which were formed in the reaction.

Conversion to the C_3 acetates was achieved by dissolving the crude product from the reaction in acetic anhydride-pyridine (1:1, 20 ml) and allowing the solution to stand at room temperature for 12 h. The solution was then poured onto crushed ice and the resultant flocculent solid extracted into ether. The combined ethereal extracts were washed with saturated aqueous sodium hydrogen carbonate solution and then several times with water before drying over anhydrous sodium sulfate. The volume of the ethereal solution was reduced *in vacuo* to 20 ml and then allowed to stand whereupon colorless needles were deposited, m.p. 187–189° (370 mg). Examination of the crystals by thin-layer chromatography indicated only one compound was present and recrystallization from ether gave an analytical sample, m.p. 189–190°. This compound was designated A and the physical and spectral data are in accord with structure 3; i.r. ($CHCl_3$): 5.83 (OAc), 6.25, 6.37 (pyridine) μ ; u.v.: λ_{max} 270 nm (3.61); $[\alpha]_D^{+83}$ (c, 1.24); n.m.r.: 9.21 (angular methyl, 3H, singlet), 8.79 (C_{21} methyl, 3H, $J_{21,20} = 7.0$ Hz, doublet), 8.40 (C_{18} methyl, 3H, singlet), 8.01 (acetate, 3H, singlet), 7.70 (C_{26} methyl, 3H, singlet), 6.71 ($C_{20}H$, 1H, $J_{20,21} = 7.0$ Hz, quartet), 3.89 (OH, 1H, broad multiplet, disappears on D_2O addition), 2.82 (C_{23} , 1H, $J_{23,24} = 8$ Hz, doublet), 2.52 (C_{24} , 1H, $J_{24,23} = 8$ and $J_{24,27} = 2$ Hz, two doublets), 1.67 (C_{27} , 1H, $J_{27,24} = 2$ Hz, doublet); mass spectrometry: m/e 451 (M^+), prominent peaks at m/e 433, 330, 147, and 121.

Anal. Calcd. for $C_{29}H_{41}O_3N$: C, 77.12; H, 9.15; O, 10.63; N, 3.10. Found: C, 76.92; H, 8.95; O, 10.46; N, 3.21.

Examination by thin-layer chromatography of the mother liquors from the crystallization of compound A indicated the presence of a further compound of similar color reaction when sprayed with antimony pentachloride-carbon tetrachloride reagent. These mother liquors were combined and chromatographed on alumina (60 g, activity III). Elution with benzene yielded unreacted 2-ethyl-5-methylpyridine initially, while further elution provided an additional compound (350 mg) which was obtained crystalline from a small volume of ether, m.p. 190–192°. Recrystallization from acetone-petroleum ether gave an analytical sample (250 mg), m.p. 190–192°. This compound was designated compound B and was slightly less polar than compound A on a silica gel chromatoplate developed with 2% methanol in chloroform; i.r. ($CHCl_3$): 5.83 (OAc), 6.25, 6.37 (pyridine) μ , u.v.: λ_{max} 265sh (3.57), 270 (3.61), 276sh nm (3.51); $[\alpha]_D^{+126}$ (c, 1.26); n.m.r.: 9.23 (angular methyl, 3H, singlet), 8.88 (C_{21} methyl, 3H, $J_{20,21} = 7.0$ Hz, doublet), 8.28 (C_{18} methyl, 3H, singlet), 8.02 (CH_3CO , 3H, singlet), 7.72 (C_{26} methyl, 3H, singlet), 6.71 (C_{20} , 1H, $J_{20,21} = 7.0$ Hz, quartet), 4.20 (OH, 1H, broad signal, disappears on D_2O addition), 2.92 (C_{23} , 1H, $J_{23,24} = 8.0$ Hz, doublet), 2.70 (C_{24} , 1H, $J_{24,23} = 8$ and $J_{24,27} = 2.0$

Hz), 1.75 (C_{27} , 1H, $J_{27,24} = 2.0$ Hz, doublet); mass spectrometry: m/e 451 (M^+), prominent peaks at m/e 433, 418, 330, 147, and 121.

Anal. Calcd. for $C_{29}H_{41}O_3N$: C, 77.12; H, 9.15; N, 3.10. Found: C, 77.19; H, 9.21; N, 3.19.

Elution with 20% ether in benzene gave additional compound A (150 mg) while two fractions appeared to contain two further compounds of similar reaction to the spray reagent when examined by thin-layer chromatography. Separation by preparative layer chromatography (20×20 cm, 0.3 mm, 1% methanol in chloroform, chromatoplate developed twice) yielded 35 mg and 8 mg of these compounds which were designated C and D, respectively. Compound C could not be induced to crystallize and an analytical sample was prepared by sublimation at 190°/0.1 mm which gave a clear glass. The spectral data were consistent with this compound being isomeric with A and B; i.r.: 5.8 (—OAc), 6.25, 6.39 (pyridine) μ ; u.v.: λ_{max} 270 nm (3.60); n.m.r.: 9.26 (angular methyl, 3H, singlet), 8.77 (C_{21} methyl, 3H, $J_{21,20} = 7.02$ Hz, doublet), 8.43 (C_{18} methyl, 3H, broad singlet), 8.01 (CH_3CO , 3H, singlet), 7.73 (C_{26} methyl, 3H, singlet), 6.90 ($C_{20}H$, 1H, $J_{20,21} = 7.0$ Hz, quartet), 2.97 (C_{23} , 1H, $J_{23,24} = 8.0$ Hz, doublet), 2.65 (C_{24} , 1H, $J_{24,23} = 8.0$ and $J_{24,27} = 2.0$ Hz, two doublets), 1.72 ($C_{27}H$, 1H, $J_{27,24} = 2.0$ Hz, doublet); mass spectrometry: m/e 451 (M^+), prominent peaks at m/e 433, 418, 330, and 120.

Anal. Calcd. for $C_{29}H_{41}O_3N$: C, 77.12; H, 9.15. Found: C, 77.05; H, 9.33.

Compound D could not be induced to crystallize but the n.m.r. and mass spectrum indicate this compound is probably isomeric with the three characterized above; i.r. ($CHCl_3$): 5.83 (OAc), 6.25, 6.37 (pyridine) μ ; u.v.: λ_{max} 270 nm (3.62); n.m.r.: 9.21 (angular methyl, 3H, singlet), 8.63 (C_{21} methyl, 3H, $J_{21,20} = 7.0$ Hz, doublet), 8.27 (C_{18} methyl, 3H, singlet), 8.01 (CH_3CO , 3H, singlet), 7.74 (C_{26} methyl, 3H, singlet), 6.89 (C_{20} , 1H, $J_{20,21} = 7.0$ Hz, quartet), 3.08 (C_{23} , 1H, $J_{23,24} = 8.0$ Hz, doublet), 2.62 (C_{24} , 1H, $J_{24,23} = 8.0$ and $J_{24,27} = 2.0$ Hz, two doublets), 1.70 (C_{27} , 1H, $J_{27,24} = 2.0$ Hz, doublet); mass spectrometry: m/e 451 (M^+), prominent peaks at m/e 433, 418, and 121.

3 β -Acetoxy-22,27-iminojerv-12(13),14(15),16,22,24,27-hexaene (4)

Aromatic 1

Compound A (118 mg) was thoroughly ground with 10% palladized charcoal (30 mg) until a homogeneous powder was obtained. This powder was heated at 200° for 7 min and the resultant solid residue washed several times with chloroform. Thin-layer chromatography of the chloroform extract indicated the product contained three major components and these were separated by preparative layer chromatography (20×20 cm, 0.4 mm, 2% methanol in chloroform). One of these three components was shown to be 2-ethyl-5-methylpyridine by n.m.r. The band which exhibited a strong fluorescence when the chromatoplate was viewed under u.v. light was extracted and yielded a light oil which crystallized from acetone-petroleum ether as needles, m.p. 167°. This compound was identified as 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (1) by its u.v. spectrum and by mixture melting point with an authentic sample. The third compound which appeared as a light orange spot when the chromatoplate was sprayed with

antimony pentachloride-carbon tetrachloride reagent was extracted to yield a light oil which could not be induced to crystallize. An analytical sample of this compound which was designated aromatic I was obtained as a clear glass after sublimation at 185°/0.1 mm. Spectral data indicated this compound possessed structure 4; i.r.: 5.82, 6.26, 6.39 μ ; u.v.: $\lambda_{\max}$ 265sh, 269 (3.73) 277 nm (3.62); $[\alpha]_D + 13^\circ$ (c, 0.84); n.m.r.: 9.10 (angular methyl, 3H, singlet), 8.41 (C₂₁ methyl, 3H, $J_{21,20} = 7.0$ Hz, doublet), 8.03 (CH₃CO, 3H, singlet), 7.90 (C₁₈ methyl, 3H, singlet), 7.80 (C₂₆ methyl, 3H, singlet), 5.61 (C₂₀, 1H, $J_{20,21} = 7.0$ Hz, quartet), 3.22-3.0 (C₁₅ or C₁₆ and C₂₃, 2H, two overlapping doublets), 2.94 (C₁₅ or C₁₆, 1H, $J = 8$ Hz, doublet), 2.72 (C₂₄, 1H, $J_{24,23} = 8.0$ and $J_{24,27} = 2$ Hz, two doublets), 1.7 (C₂₇H, 1H, $J_{27,24} = 2$ Hz, doublet); mass spectrometry: m/e 431, prominent peaks at 416, 402.

Mol. Wt. Calcd. for C₂₉H₃₇O₂N: 431.2824. Found (high resolution mass measurement): 431.2809.

Aromatic II

Compound B (80 mg) was ground with 10% palladized charcoal (20 mg) until a homogeneous powder was formed. This powder was heated at 200° for 7 min and the solid residue washed several times with chloroform. Thin-layer chromatographic examination of the chloroform extract indicated the major component was a new compound which appeared as a light orange spot when the chromatoplate was sprayed with antimony pentachloride-carbon tetrachloride reagent. This compound was separated from the reaction mixture by preparative layer chromatography (20 × 20 cm, 0.4 mm, 1% methanol in chloroform, developed twice) as a light oil (60 mg) which could not be induced to crystallize. An analytical sample of the compound, which was designated 'aromatic II', was obtained as a clear glass after sublimation at 185°/0.01 mm; i.r.: 5.82, 6.26, 6.39 μ ; u.v.: $\lambda_{\max}$ 265sh, 269 (3.73), 277 nm (3.62); n.m.r.: 9.10 (angular methyl, 3H, singlet), 8.40 (C₂₁ methyl, 3H, $J_{21,20} = 7$ Hz, doublet), 8.02 (CH₃CO, 3H, singlet), 7.89 (C₁₈ methyl, 3H, singlet), 7.80 (C₂₆ methyl, 3H, singlet), 5.58 (C₂₀, 1H, $J_{20,21} = 7.0$ Hz, quartet), 3.14 (C₂₃, 1H, $J_{23,24} = 8$ Hz, doublet), 3.09 (C₁₅ or C₁₆, 1H, $J = 8$ Hz, doublet), 2.91 (C₁₅ or C₁₆, 1H, $J = 8$ Hz, doublet), 2.75 (C₂₄, 1H, $J_{24,23} = 8$ and $J_{24,27} = 2$ Hz, two doublets), 1.73 (C₂₇, 1H, $J_{27,24} = 2$ Hz, doublet); mass spectrometry: m/e 431 (M⁺), prominent peaks at m/e 416, 402.

Mol. Wt. Calcd. for C₂₉H₃₇O₂N: 431.2824. Found (high resolution mass measurement): 431.2809.

N-Acetyl-22,27-iminojerva-12(13),14(15),16-triene-3 β -ol (5)

Isomers i-iv

Aromatic compound II (50 mg) was dissolved in glacial acetic acid (10 ml) and the solution hydrogenated at 20° and 45 p.s.i. over Adams' catalyst (PtO₂, 20 mg) for 3 h. The mixture was filtered to remove the catalyst which was carefully washed with additional acetic acid (5 ml). The combined filtrates were reduced in volume to 2 ml *in vacuo*, then diluted with water (20 ml) and made basic with ammonia solution. The resultant suspension was extracted with methylene chloride (3 × 10 ml) before drying over anhydrous sodium sulfate. Thin-layer chromatography revealed the presence of four compounds of similar R_f value and color reaction with various spray reagents. These compounds were designated i-iv in order of de-

creasing R_f value, (silica gel G, 5% methanol in chloroform) and were separated by careful preparative layer chromatography (20 × 20 cm, 0.3 mm, 1% methanol in chloroform, plates developed three times). The bands corresponding to the four compounds were delineated by inspection of the developed plates under u.v. light. Extraction of the various bands and removal of the solvent (methanol-chloroform 1:1) gave each of the compounds as a clear oil. None of the compounds could be induced to crystallize.

All four compounds were separately converted via the 3-O,N-diacetates (13) to the N-acetyl derivatives 12 (since a sample of N-acetyl-5 α ,6-dihydroverarine was available for comparison) as outlined for one of the isomers below.

Compound ii was dissolved in pyridine-acetic anhydride (2 ml, 1:1) and allowed to stand for 12 h at 20°. The solution was then poured into ice water (5 ml) and the resulting suspension extracted with methylene chloride (3 × 3 ml). The combined extracts were washed with saturated aqueous sodium hydrogen carbonate (10 ml) and then with water (2 × 5 ml), before drying over anhydrous sodium sulfate. Removal of the methylene chloride *in vacuo* gave a light oil which crystallized from ether (the diacetates of compounds i, iii, and iv were not obtained crystalline) as needles, m.p. 203-205°; i.r. (CHCl₃): 5.80 (OAc), 6.19 (Nac) μ ; u.v.: $\lambda_{\max}$ 268 (2.7), 277 nm (2.7).

The diacetate (20 mg) was refluxed with 0.1 M potassium hydroxide in methanol (5 ml) for 1 h, the solution cooled, and diluted with water (20 ml). The aqueous suspension was extracted with methylene chloride (3 × 5 ml) and the combined extracts washed with water (2 × 5 ml) prior to drying over anhydrous sodium sulfate. Evaporation of the solvent *in vacuo* gave a light oil which was readily crystallized from anhydrous ether. (All four N-acetyl derivatives were obtained crystalline via this procedure.)

The N-acetyl derivatives of compounds i and ii had R_f values comparable with that of N-acetyl-5 α ,6-dihydroverarine whereas those of the N-acetyl derivatives of compounds iii and iv were slightly smaller. All compounds showed similar color reactions when the chromatoplate was sprayed with various reagents. The N-acetyl derivatives were characterized as follows.

Compound i, N-acetate; prisms, m.p. 249-250° (6 mg); i.r. (CHCl₃): 6.20, 9.70 μ ; u.v.: $\lambda_{\max}$ 268 (2.68), 277 nm (2.66); $[\alpha]_D + 36^\circ$ (c, 0.605); n.m.r.: 9.07 (angular methyl, 3H, singlet), 8.19 (CH₃CON, 3H, singlet), 7.74 (C₁₈ methyl, 3H, singlet), 3.15 (C₁₅ or C₁₆, $J = 8$ Hz, doublet), 2.96 (C₁₅ or C₁₆, $J = 8$ Hz, doublet); mass spectrometry: m/e 437 (M⁺), prominent peaks at m/e 297, 140, 98.

Mol. Wt. Calcd. for C₂₉H₄₃O₂N: 437.329. Found (high resolution mass measurement): 437.325.

Compound ii, N-acetate; prisms, m.p. 263-264° (17 mg); i.r. (CHCl₃): 6.19, 9.70 μ ; u.v.: $\lambda_{\max}$ 268 (2.65), 277 nm (2.61); $[\alpha]_D + 26^\circ$ (c, 1.71); mass spectrometry: m/e 437 (M⁺), prominent peaks at m/e 298, 297, 140, 98.

Compound iii, N-acetate; rosettes, m.p. 270-274° (5 mg); i.r. (CHCl₃): 6.20, 9.70 μ ; u.v.: $\lambda_{\max}$ 268, 277 nm; mass spectrometry: m/e 437 (M⁺), prominent peaks at m/e 297, 140, 98.

Compound iv, N-acetate; long needles, m.p. 260-261° (10 mg); i.r. (CHCl₃): 6.20, 9.70, 9.80 μ ; u.v.: $\lambda_{\max}$ 268, 277 nm; mass spectrometry: m/e 437 (M⁺), prominent peaks at m/e 298, 297, 140, 98.

Isomers v-viii

Aromatic compound I (50 mg) was dissolved in glacial acetic acid (10 ml) and the solution shaken with Adams' catalyst (PtO_2 , 20 mg) in an atmosphere of hydrogen at 45 p.s.i. and 20° for a period of 3 h. The mixture was filtered to remove the catalyst which was washed with a further 10 ml of glacial acetic acid and the filtrates combined. The volume of the acetic acid was reduced to 2 ml by use of a rotary evaporator before dilution with water (20 ml). The aqueous acid solution was made basic with dilute ammonia and the resulting suspension extracted several times with methylene chloride (3×10 ml) prior to drying over anhydrous sodium sulfate. Thin-layer chromatographic investigation of the light oil (45 mg) obtained by removal of the methylene chloride indicated the presence of four compounds, of similar R_f value and color reaction to the spray reagent. These compounds were numbered v-viii in order of decreasing R_f value and were separated by careful preparative layer chromatography (silica gel G 20×20 cm, 0.3 mm, 1% methanol in chloroform, plates developed three times). The compounds were obtained as colorless oils which could not be crystallized. Conversion of these compounds via the 3-*O,N*-diacetates to the *N*-acetyl derivatives was accomplished as outlined above for the 3 β -acetoxy-22,27-iminojerva-12(13),14(15),16-triene isomers i-iv obtained from aromatic compound II. None of the *N*-acetyl derivatives of compounds v-viii could be induced to crystallize although compounds v and vi had R_f values comparable with *N*-acetyl-5,6-dihydroverarine. These compounds were not fully characterized but their spectral properties were in accord with structure 5.

Compound v, N-acetate: i.r.: 6.19, 9.28 μ ; u.v.: λ_{max} 267, 276 nm; mass spectrometry: m/e 437 (M^+), prominent peaks at m/e 298, 297, 98.

Spectral properties for the *N*-acetyl derivatives of compounds vi-viii are virtually the same as for the acetate of v and no additional data were obtained for any of these compounds.

Hydrogenation of 3-O,N-Diacetylverarine

The 3-*O,N*-diacetylverarine utilized in this reaction was prepared from veratramine (1) and had the following physical constants, m.p. $189-190^\circ$ (lit. (1) m.p. $189-190^\circ$); $[\alpha]_D -23 \pm 2^\circ$ (ethanol).

A solution of 3-*O,N*-diacetylverarine (190 mg) in glacial acetic acid (5 ml) was stirred with Adams' catalyst (PtO_2 , 60 mg) in an atmosphere of hydrogen at 20° . After 14 h, 9.1 ml of hydrogen had been consumed and the mixture was filtered to remove the catalyst which was washed with a further 10 ml of acetic acid. The combined filtrates were reduced in volume (to 2 ml) *in vacuo* and diluted with water (30 ml) to give a white precipitate which was taken up in ether. The ethereal phase was washed with water (3×10 ml) before drying over anhydrous sodium sulfate. Thin-layer chromatographic examination on both silica gel and alumina using a variety of solvents indicated that a single new compound had been formed.

Removal of the ether *in vacuo* gave an oil which could not be induced to crystallize. A small scale investigation revealed that after selective hydrolysis of the 3-*O*-acetate the product appeared as two components of similar R_f value on a silica gel chromatoplate. Consequently 3-*O,N*-diacetyl-5,6-dihydroverarine (170 mg) was refluxed

with 0.1 *N* methanolic potassium hydroxide (5 ml) for 1 h, the solution cooled, and then diluted with water (20 ml). The resulting aqueous suspension was extracted with methylene chloride (3×10 ml) and the combined extracts washed with water (3×10 ml) before drying over anhydrous sodium sulfate. Removal of the solvent *in vacuo* gave a light oil which on trituration with ether gave crystals, m.p. $190-225^\circ$. Thin-layer chromatography showed the crystalline product to consist of two compounds which were separated by preparative layer chromatography on silica gel (20×20 cm, 0.4 mm, 2% methanol in chloroform). The least polar compound (75 mg) was tentatively assigned the 5 β configuration on the basis of its n.m.r. spectrum. Recrystallization from ether gave an analytical sample of *N*-acetyl-5 β ,6-dihydrovera-

rine (20), m.p. $198-199^\circ$; i.r. (CHCl_3): 6.21 ($-\text{NC}-\text{CH}$) μ ; u.v.: λ_{max} 267 (2.74), 276 nm (2.74); n.m.r.: 9.02 (C_{26} methyl, 3H, $J_{26,25} = 7$ Hz, doublet), 8.94 (angular methyl, 3H, singlet), 8.82 (C_{21} methyl, 3H, $J_{21,20} = 8$ Hz, doublet), 8.14 (CH_3CON , 3H, singlet), 7.71 (C_{18} methyl, 3H, singlet), 6.45 (C_{20} , 1H, $J_{20,21} = 8$ and $J_{20,22} = 10$ Hz, multiplet), 5.97 ($\text{H}-\text{C}-\text{OAc}$, 1H, multiplet), 3.12 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet), 2.96 (C_{15} or C_{16} , $J = 8$ Hz, doublet); mass spectrometry: m/e 437 (M^+), prominent peaks at m/e 297, 140, 98.

Anal. Calcd. for $\text{C}_{29}\text{H}_{45}\text{O}_2\text{N}$: C, 79.58; H, 9.90; N, 3.20. Found: C, 79.51; H, 10.02; N, 3.25.

The more polar compound was crystallized from ether as prisms, m.p. $249-250^\circ$. This compound was tentatively assigned the 5 α configuration on the basis of its n.m.r. spectrum and an analytical sample of *N*-acetyl-5 α ,6-dihydroverarine (11) was prepared by sublimation at 220° /

0.01 mm, as prisms, m.p. $249-250^\circ$; i.r.: 6.22 ($-\text{NC}-\text{CH}_2$), 12.23 μ ; u.v.: λ_{max} 267 (2.69), 276 nm (2.68); $[\alpha]_D +41^\circ$ (c, 0.961); n.m.r.: 9.06, 9.02 (angular and C_{26} methyl, 6H, singlet and doublet), 8.19 (CH_3CON , 3H, singlet), 7.72 (C_{18} methyl, 3H, singlet), 3.13 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet), 2.99 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet); mass spectrometry: m/e 437 (M^+), prominent peaks at m/e 394, 297, 140.

Anal. Calcd. for $\text{C}_{29}\text{H}_{45}\text{O}_2\text{N}$: C, 79.58; H, 9.90. Found: C, 79.32; H, 9.99.

N-Acetyl-3-keto-5 α ,6-dihydroverarine (23)

A solution of *N*-acetyl-5 α ,6-dihydroverarine (11; 212 mg) in acetone (75 ml) was treated with Jones' reagent (0.3 ml) and allowed to stand at 20° for 2 min. Isopropanol (2 ml) was added to remove the excess oxidant and the mixture diluted with water (600 ml). The aqueous acetone solution was extracted with methylene chloride (3×30 ml) and the combined extracts washed with water (3×30 ml) before drying over anhydrous sodium sulfate. Evaporation of the methylene chloride gave a colorless oil (191 mg) which could not be crystallized but appeared homogeneous when examined by thin-layer chromatography. An analytical sample was obtained as a clear glass after sublimation at $185^\circ/0.01$ mm; i.r.: 5.89 (saturated car-

bonyl), 6.14 ($-\text{NC}-\text{CH}_2$) μ ; u.v.: λ_{max} 267 (2.73), 276

nm (2.71); $[\alpha]_D + 66^\circ$ (c, 1.15); o.r.d. $[\phi]_{505}^{\text{peak}} + 1090^\circ$, $[\phi]_{270}^{\text{trough}} + 115^\circ$, $[\phi]_{230}^{\text{peak}} + 3910^\circ$; n.m.r.: 9.03 (C_{26} methyl, 3H, $J_{26,25} = 7.0$ Hz, doublet), 8.88 (angular methyl, 3H, singlet), 6.45 (C_{20} , 1H, $J_{20,21} = 7.5$, $J_{20,22} = 9.0$ Hz, multiplet), 3.13 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet), 2.95 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet); mass spectrometry: m/e 435 (M^+), prominent peaks at m/e 392, 295, 140, 98.

Mol. Wt. Calcd. for $C_{29}H_{41}O_2N$: 435.313. Found (high resolution mass measurement): 435.312.

N-Acetyl- Δ^4 -3-keto-5,6-dihydroverarine (28)

A solution of *N*-acetyl-3-keto-5 α ,6-dihydroverarine (162 mg, 0.372 mmol) in acetic acid (6 ml) containing hydrogen bromide (60 mg, 0.744 mmol) was placed in a dry round-bottom flask. Bromine (130 mg, 0.8 mmol) in acetic acid (5 ml) was added slowly and the mixture stirred at 20° for 20 min and then diluted with water (25 ml). The resulting heavy white precipitate was taken up in methylene chloride and the extract washed with water (3×10 ml) prior to drying over anhydrous sodium sulfate. Removal of the solvent *in vacuo* gave a colorless gum (225 mg) which was shown by thin-layer chromatography to consist of one major compound. This crude product was immediately subjected to the conditions of Evans (12) for introduction of the 4,5-double bond as outlined below. Bromine (0.26 ml) was added to acetone (7.5 ml) at 0° and the solution stirred until the color disappeared. Sodium carbonate (0.7 g) was then added and the mixture was stirred for a further 30 min, then filtered directly into hot acetone containing sodium iodide (7.0 g). This solution was refluxed for 15 min during which time a precipitate of sodium bromide was formed.

A portion (6 ml) of the supernatant of this hot solution was added to a round bottom-flask containing 2,4-dibromo ketone (220 mg) and the solution refluxed for 2½ h. During this time more sodium bromide precipitated, indicative of the exchange of the 2-bromine for an iodine atom. Oxalic acid (220 mg) was added as a fine powder and the solution refluxed for a further hour before cooling. The reaction mixture was diluted with ethyl acetate (20 ml) and filtered. This filtrate was washed with water (2×10 ml), saturated aqueous sodium hydrogen carbonate (2×10 ml), and water (2×10 ml). The ethyl acetate was decolorized by adding zinc dust (600 mg) and acetic acid (0.2 ml) and then shaking. When the solution had attained a light yellow color the zinc dust was removed by filtration and the ethyl acetate filtrate washed with water (2×10 ml), sodium hydrogen carbonate solution (2×10 ml), and water (2×10 ml) before drying over anhydrous sodium sulfate.

Removal of the ethyl acetate *in vacuo* gave a light yellow gum (158 mg) which was examined by thin-layer chromatography. The spot on the chromatoplate corresponding to the major product exhibited an intense fluorescence under u.v. light and appeared purple when the plate was sprayed with antimony pentachloride-carbon tetrachloride reagent. Preparative layer chromatography on silica gel (20×60 cm, 0.4 mm, developed twice with 1% methanol in chloroform) gave this compound as a light gum (110 mg, 50% yield) which was not crystallized. An analytical sample was prepared by sublimation at $180^\circ/0.01$ mm which gave a light yellow glass; i.r.: 6.03 (α , β -unsaturated carbonyl), 6.14 (N-Ac) μ ; $[\alpha]_D + 74^\circ$ (c, 1.26); n.m.r.: 9.02 (C_{26} methyl, 3H, $J_{26,25} = 7$ Hz, dou-

blet), 8.72 (angular methyl, 3H, singlet), 8.18 ($CH_3C=N$, 3H, singlet), 6.45 (C_{20} , 1H, $J_{20,21} = 7.5$, $J_{20,22} = 10$ Hz, multiplet), 4.22 (C_4 , 1H, singlet), 3.10 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet), 2.94 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet); mass spectrometry: m/e 433 (M^+), prominent peaks at m/e 390, 293, 140, 98.

Mol. Wt. Calcd. for $C_{29}H_{39}O_2N$: 433.2980. Found (high resolution mass measurement): 433.2974.

N-Acetylverarine (30)

A solution of *N*-acetyl- Δ^4 -3-keto-5,6-dihydroverarine (100 mg) in isopropenyl acetate (3 ml) containing 0.5% sulfuric acid was refluxed for 1 h. Anhydrous sodium acetate (60 mg) was then added and most of the isopropenyl acetate removed by use of a rotary evaporator. The residue was diluted with methylene chloride (10 ml) and then filtered. Thin-layer chromatography of the filtrate indicated the complete absence of (23) and the formation of a less polar compound. Evaporation of the methylene chloride gave an oil (100 mg) the i.r. spectrum of which exhibited a carbonyl band at 5.75μ indicative of an enol acetate.

This oil was dissolved in a mixture of methanol (10 ml) and ether (3 ml) and heated to reflux. During the refluxing a methanolic solution of sodium borohydride (150 mg in 5 ml) was added slowly over a period of 15 min. The solution was refluxed for a further 1 h and then concentrated hydrochloric acid (1 ml) was added and the refluxing continued with stirring for 30 min. During this final period vigorous stirring was necessary to overcome severe bumping.

The reaction mixture was cooled and then diluted with ether (50 ml). The ethereal solution was washed with water and then dried over anhydrous sodium sulfate. Removal of the solvent gave a light oil (70 mg) which was shown to contain a compound of identical R_f value to that of *N*-acetylverarine. Separation by preparative layer chromatography (20×20 cm, 0.3 mm, 2% methanol in chloroform) and extraction of the band corresponding to this product yielded an oil (30 mg). This compound was shown to be identical with *N*-acetylverarine by thin-layer chromatography and superimposable i.r. and n.m.r. spec-

tra; i.r. ($CHCl_3$): 6.19 ($NC=CH_2$) μ ; u.v.: λ_{max} 268 (2.76) 277 nm (2.65); n.m.r.: 9.03 (C_{26} methyl, 3H, $J_{26,25} = 7$ Hz, doublet), 8.87 (angular methyl, 3H, singlet), 8.18 (CH_3CON , 3H, singlet), 4.56 (C_6 1H, multiplet), 3.12 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet), 2.94 (C_{15} or C_{16} , 1H, $J = 8$ Hz, doublet); mass spectrometry: m/e 435 (M^+), prominent peaks at m/e 295, 140, 98.

Verarine (31)

A solution of *N*-acetylverarine (20 mg) in ethylene glycol (5 ml) containing 10% potassium hydroxide was refluxed for 12 h. The reaction mixture was cooled, diluted with water (20 ml), and extracted with methylene chloride (3×5 ml). Examination of the extract by thin-layer chromatography indicated the presence of *N*-acetylverarine and a more polar compound which had the same R_f value and color reaction to the spray reagent, as an authentic sample of verarine. The more polar compound was separated by preparative layer chromatography (20×20 cm, 0.3 mm, 5% methanol in chloroform) as a light oil

(8 mg) which crystallized from ether. This compound was shown to be identical with verarine by thin-layer chromatography, i.r., and n.m.r. comparison; i.r. (CHCl_3): 9.57 μ ; u.v.: λ_{max} 267 (2.74) 276 nm (2.73); n.m.r.: 9.15 (C_{26} methyl, 3H, $J_{26,25} = 6$ Hz, doublet), 8.88 (angular methyl, 3H, singlet), 7.73 (C_{18} methyl, 3H, singlet), 4.55 (C_6 , 1H, broad doublet), 3.02 (C_{15} and C_{16}H , 2H, singlet); mass spectrometry: m/e 393 (M^+), prominent peaks at m/e 392, 376, 295, 284, 256, 98.

Mol. Wt. Calcd. for $\text{C}_{27}\text{H}_{39}\text{ON}$: 392.2953. Found (high resolution mass measurement): 392.2922.

3-Methylbut-3-enal Diethyl Acetal (40)

This compound was prepared according to the procedure of Cornforth and Firth (23) with small modifications as indicated. The additional data obtained in our investigations in this and the next few experiments are also provided. Triethyl orthoformate (45 g) and magnesium (17.5 g) were stirred and heated at 60° . 2-Methylallyl chloride (24.5 g) was then added gradually (about 2.5 h) and the reaction mixture left overnight. The flask was cooled in ice and saturated aqueous ammonium chloride (20 ml) was added dropwise until the mixture set solid; the cake was filtered and washed well with ether. The filtrate on work-up afforded the acetal (40, 19 g), b.p. $60\text{--}61^\circ/20$ mm (lit. (25) b.p. $60^\circ/19$ mm). The compound was found to be pure by v.p.c. examination using a FFAP column; i.r. (film): 6.25 μ ; n.m.r.: 8.85 ($2 \times \text{CH}_3\text{--CH}_2$, 6H, triplet), 8.25 ($\text{C}_2\text{--CH}_3$, 3H, singlet), 7.7 ($\text{C}_3\text{--CH}_2$, 2H, doublet, $J = 6.0$ Hz), 6.5 (CH_2 's of $\text{--O--CH}_2\text{--CH}_3$, 4H, multiplet), 5.4 (C_4H , 1H, triplet), 5.2 ($\text{CH}_2\text{=C}$, 2H, singlet).

3,4-Epoxy-3-methylbutanal Diethyl Acetal (41)

A solution of 3-methylbut-3-enal diethyl acetal (40, 6.6 g) in anhydrous ether (10 ml) was cooled in ice and treated gradually with a solution of *m*-chloroperbenzoic acid (9 g) in ether (30 ml). The reaction mixture was then allowed to warm up and kept at 30° by occasional cooling until the reaction subsided. Next day the reaction mixture was washed repeatedly with saturated sodium bicarbonate solution until the whole of *m*-chlorobenzoic acid was removed. The ether layer was separated and dried over magnesium sulfate. The solvent was removed under reduced pressure and distillation of the residual liquid afforded the epoxide (41) as a colorless oil (4.1 g), b.p. $82\text{--}86^\circ/18$ mm (lit. (22) b.p. $83\text{--}84^\circ/17$ mm). The compound was found to be homogeneous by v.p.c. examination; i.r. (film): 10.0, 10.5, 12.5 μ (epoxide); n.m.r.: 8.85 ($2 \times \text{CH}_3\text{--CH}_2\text{--O}$, 6H, triplet), 8.7 ($\text{C}_3\text{--CH}_2$, 3H, singlet), 8.2 ($\text{C}_2\text{--CH}_2$, 2H, doublet, $J = 7.0$ Hz), 7.5

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 $\text{CH}_2\text{--C--}$, 2H, AB quartet, $J_{A,B} = 5.5$ Hz), 6.5 (CH_2 's of $\text{--O--CH}_2\text{--CH}_3$, 4H multiplet), 5.4 (C_1H , 1H, triplet).

3-Methylfuran (42)

A mixture of 3,4-epoxy-3-methylbutanal diethyl acetal (41, 5.0 g) and 0.1 *N* sulfuric acid (500 ml) were heated under a fractionating column for 3 h, the methylfuran and ethanol being removed intermittently by distillation. The distillate was washed once with half-saturated aqueous calcium chloride (10 ml) and twice with saturated aqueous ammonium chloride, dried over sodium, and redistilled to

give pure 3-methylfuran (1.1 g); b.p. $65\text{--}66^\circ$ (lit. (22) b.p. $65\text{--}65.5^\circ$); i.r. (chloroform): 6.67, 8.33, 11.2 μ ; n.m.r.: 8.0 ($\text{C}_3\text{--CH}_3$, 3H, singlet), 3.85 (C_4H , 1H, singlet), 2.87 (C_2H , 1H, broad singlet), 2.75 (C_5H , 1H, broad singlet).

Propionylation of 3-Methylfuran

3-Methylfuran (1 g) and propionic anhydride (1.6 g) were mixed and to the mixture 2 drops of orthophosphoric acid (85%) were added. The mixture was stirred vigorously and the heat generated was removed by cooling. Gradually the reaction mixture acquired a dark red color. It was then heated to $60\text{--}65^\circ$ and maintained at this temperature for 2 h. The reaction mixture was then cooled and stirred with water (2 ml) for 1 h. The dark organic layer was separated and again stirred with saturated sodium carbonate solution (5 ml) for 24 h. It was then washed thoroughly with water, extracted with ether (2×5 ml), and dried over magnesium sulfate. The solvent was removed under reduced pressure and the residual reddish brown oil obtained was distilled *in vacuo* in a hot box. A colorless fragrant liquid (0.5 g) was collected, b.p. $125\text{--}130^\circ/25$ mm. The i.r. spectrum (film) of the product showed a double carbonyl absorption (~ 5.88 μ). Examination of this material by v.p.c. using a FFAP column (172°) indicated that it was a mixture of two compounds in the ratio 70:30, with retention times 1.8 and 2.8 min, respectively. These were separated by v.p.c. using a FFAP column (172°). The major component was characterized as 2-propionyl-3-methylfuran (43), b.p. $77\text{--}80^\circ/5$ mm; i.r. (film): 5.99, 6.65, 11.3 (furan) μ ; u.v.: λ_{max} 278, 230 nm; n.m.r.: 8.85 ($\text{CH}_3\text{--CH}_2$, 3H, triplet), 7.65 ($\text{C}_3\text{--CH}_3$, 3H, singlet), 7.2 ($\text{--CO--CH}_2\text{--CH}_3$, 2H, quartet), 3.65 (C_4H , 1H, doublet, $J_{4,5} = 2.5$ Hz), 2.65 (C_5H , doublet, $J_{4,5} = 2.5$ Hz); mass spectrometry: m/e 138 (M^+), prominent peak at m/e 109.

Anal. Calcd. for $\text{C}_8\text{H}_{10}\text{O}_2$: C, 69.56; H, 7.24. Found: C, 69.71; H, 7.27.

The second component was characterized as 2-propionyl-4-methylfuran (44), b.p. $78\text{--}82^\circ/5$ mm; i.r. (film): 5.97, 6.65, 11.3 μ ; u.v.: λ_{max} 280, 230 nm; n.m.r.: 8.85 ($\text{CH}_3\text{--CH}_2$, 3H, triplet), 7.9 (C_4CH_3 , 3H, singlet), 7.2 ($\text{--CO--CH}_2\text{--CH}_3$, 2H, quartet), 3.05 (C_3H , 1H, singlet), 2.75 (C_5H , 1H, broad singlet); mass spectrometry: m/e 138 (M^+), prominent peak at m/e 109.

Anal. Calcd. for $\text{C}_8\text{H}_{10}\text{O}_2$: C, 69.56; H, 7.24. Found: C, 69.68; H, 7.26.

Methyl 5,5-Dimethoxy-3-methyl-2,3-epoxypentanoate (45)

This compound was prepared according to the procedure of Burness (25).

Methyl 3-Methyl-2-furoate (46)

This compound was prepared according to the procedure of Burness (25); i.r. (chloroform): 5.80 μ ; u.v.: λ_{max} 252 nm; n.m.r.: 7.65 ($\text{C}_3\text{--CH}_3$, 3H, singlet), 6.2 (COOCH_3 , 3H, singlet), 3.68 (C_4H , 1H, doublet, $J = 2.0$ Hz), 2.6 (C_5H , 1H, doublet, $J = 2.0$ Hz).

Methyl 3-Methyl-5-propionyl-2-furoate (47)

Methyl 3-methyl-2-furoate (25.6 g, 0.2 mol) was dissolved in propionic anhydride (75 ml) and orthophosphoric acid (7.0 g) was added to it with vigorous stirring. The heat generated was removed by cooling with ice. The reaction mixture was then stirred at $65\text{--}70^\circ$ for 48 h. It

was then cooled and stirred into water. The dark brown organic layer was extracted with chloroform and the chloroform extract was washed with bicarbonate solution until neutral to litmus and dried over sodium sulfate. The solvent was distilled off under reduced pressure. The unreacted starting materials were removed by distillation *in vacuo* and the semisolid residue was distilled at 120°/0.3–4 mm. A pale yellow crystalline material was collected in the receiver (23.5 g), m.p. 109–111°. It was recrystallized from aqueous ethanol (70%), colorless shining needles, m.p. 113–114°; i.r. (chloroform): 5.73, 5.88 μ ; u.v.: $\lambda_{\max}$ 280, 285sh, 212 nm; n.m.r.: 8.8 (—CH₂—CH₃, 3H, triplet), 7.65 (C₃CH₃, 3H, singlet), 7.2 (—CO—CH₂—, 2H, quartet), 6.1 (—COOCH₃, 3H, singlet), 3.0 (C₆H, 1H, singlet); mass spectrometry: *m/e* 196 (M⁺), prominent peaks at *m/e* 167, 138, 123, 109.

Anal. Calcd. for C₁₀H₁₂O₄: C, 61.22; H, 6.12. Found: C, 61.01; H, 5.97.

3-Methyl-5-propionyl-2-furoic Acid (48)

A solution of methyl 3-methyl-5-propionyl-2-furoate (47, 9.8 g) in aqueous sodium hydroxide (10%, 100 ml) was heated under reflux for 1.5 h. The solution was then cooled and acidified with concentrated hydrochloric acid and stirred for a few minutes. The product which separated as a brown granular solid was collected by filtration. It was washed with a little ice cold water and dried *in vacuo*. Crystallization from methanol afforded pale yellow shining prisms (7.6 g), m.p. 179–181°; i.r. (chloroform): 5.67, 5.88 μ ; u.v.: $\lambda_{\max}$ 282, 212 nm; n.m.r.: 8.8 (CH₃—CH₂—, 3H, triplet), 7.6 (C₃CH₃, 3H, singlet), 7.0 (—CH₂—CO—, 2H, quartet), 2.85 (C₄H, 1H, singlet), —0.2 (—COOH, 1H, singlet); mass spectrometry: *m/e* 182 (M⁺), prominent peak at *m/e* 137.

Anal. Calcd. for C₉H₁₀O₄: C, 59.33; H, 5.49. Found: C, 59.21; H, 5.50.

2-Propionyl-4-methylfuran (44)

A mixture of 3-methyl-5-propionyl-2-furoic acid (9.1 g, 0.05 mol), anhydrous quinoline (20 ml), and powdered copper (2.0 g) was heated 200–210° under a nitrogen atmosphere for 3 h. The evolution of carbon dioxide ceased by this time and the reaction mixture was extracted with chloroform (50 ml). The chloroform extract was filtered to remove the copper powder and washed successively with 1 *N* hydrochloric acid (3 × 50 ml), water (100 ml), and finally with sodium bicarbonate solution until neutral to litmus. It was then dried over sodium sulfate and the solvent was distilled off under reduced pressure. The residual dark oil was distilled using a short fractionating column. The fraction boiling at 78–82°/5 mm was collected as a colorless fragrant liquid (5.4 g). Examination of the compound by t.l.c. and v.p.c. indicated that it was pure. The physical and spectral data of the compound were identical with those of 44 obtained by the propionylation of 3-methylfuran.

2-Ethyl-3-hydroxy-5-methylpyridine (49)

2-Propionyl-4-methylfuran (44; 1.0 g) and aqueous ammonia (11 *N*, 25 ml), were heated in a sealed tube for 20 h (18). The reaction mixture was filtered off the residual solid matter and the filtrate was evaporated to dryness under a stream of nitrogen. The residue was extracted with chloroform and dried. The solvent was removed under reduced pressure and the dark brown semisolid residue dis-

tilled at 150°/0.3–5 mm. A small quantity of pale yellow liquid which collected in the receiver soon solidified into shining prisms (202 mg), m.p. 140–144°. Crystallization from chloroform provided an analytical sample as colorless shining prisms, m.p. 145–147°; i.r. (chloroform): 3.11, 6.29 μ ; u.v.: $\lambda_{\max}$ (log ϵ): 287 (3.95), 225sh nm; n.m.r.: 8.75 (CH₃—CH₂—, 3H, triplet), 7.8 (C₅CH₃, 3H, singlet), 7.1 (—CH₂—CH₃, 2H, quartet), 2.9 (C₄H, 1H, singlet), 2.1 (C₆H, 1H, singlet), 1.0 (C₃OH, 1H, multiplet); mass spectrometry: *m/e* 137 (M⁺), prominent peaks at *m/e* 136, 121, 94, 93.

Anal. Calcd. for C₈H₁₁NO: C, 70.07; H, 8.02. Found: C, 70.11; H, 8.10.

2-Ethyl-3-O-benzyl-5-methylpyridine (50)

2-Ethyl-5-methyl-3-hydroxypyridine (274 mg, 0.002 mol) was dissolved in aqueous sodium hydroxide (5%, 10 ml) and benzyl chloride (240 mg, 0.002 mol) was added to it. The mixture was heated at 100° for 2.5 h. During this time the benzyl chloride gradually dissolved and a pale yellow oil separated in the flask. The reaction mixture was cooled and extracted with chloroform (2 × 10 ml). The chloroform extract was washed with water until neutral to litmus and dried over sodium sulfate. The residual oil obtained after the removal of the solvent was distilled *in vacuo*. A colorless oil collected in the receiver (228 mg), b.p. 118–120°/0.4–5 mm. Thin-layer chromatography on alumina indicated that the compound was pure; i.r. (film): 6.27, 7.87, 9.52 μ ; u.v.: $\lambda_{\max}$ 285, 225sh nm; n.m.r.: 8.8 (CH₃—CH₂—, 3H, triplet), 7.75 (C₅CH₃, 3H, singlet), 7.2 (—CH₂—CH₃, 2H, quartet), 5.0 (—O—CH₂—C₆H₅, 2H, singlet), 3.15 (C₄H, 1H, singlet), 2.65 (C₆H₅—CH₂—O—, 5H, singlet), 2.1 (C₆H, 1H, singlet).

2-Ethyl-3-methoxy-5-methylpyridine (51)

A solution of 2-ethyl-3-hydroxy-5-methylpyridine (2.74 g, 0.02 mol) in methanol (40 ml) containing 10% water was chilled in an ice-salt bath. To this was added a solution of diazomethane (4.2 g, 0.1 mol) in ether (175 ml) with constant swirling from a dropping funnel, the stem of which projected below the surface of the liquid. A brisk effervescence accompanied the addition which was interrupted several times in order to boil off the accumulated ether. The latter procedure serves to keep the polarity of the solution at a maximum. After being allowed to stand overnight, the methanolic solution was acidified with hydrochloric acid and most of the ether and methanol removed by concentration on the steam bath. The resulting syrup was diluted with water and the solution exhausted with ether. It was then made alkaline with sodium hydroxide pellets and again extracted with ether (3 × 100 ml). This second extract was dried over potassium carbonate, the ether removed by distillation, and the residual oil distilled *in vacuo*. The product (2.01 g) was a colorless oil, b.p. 100°/10 mm.

Some of the starting material was recoverable by the following procedure. The alkaline solution after exhaustion with ether was neutralized and evaporated to dryness followed by extraction with chloroform. Removal of the chloroform afforded a dark brown solid (480 mg) which was identified as 2-ethyl-3-hydroxy-5-methylpyridine (49).

The methyl ether was characterized as follows; i.r. (film): 6.26, 6.41, 7.91, 9.52 μ ; u.v.: $\lambda_{\max}$ (log ϵ): 283 (3.86), 225sh nm; n.m.r.: 8.8 (CH₃—CH₂—, 3H, triplet),

7.7 (C_3CH_3 , 3H, singlet), 7.25 ($-CH_2-CH_3$, 2H, quartet), 6.25 ($-OCH_3$, 3H, singlet), 3.2 (C_4H , 1H, singlet), 2.15 (C_6H , 1H, singlet).

Anal. Calcd. for $C_9H_{13}NO$: C, 71.52; H, 8.60. Found: C, 71.47; H, 8.64.

23-Methoxy-22,27-iminojerva-12(13),22,24,27-tetraene-3 β -17-diol(s) (53)

An ether solution (7.0 ml) of 2.0 M methyl lithium was added to anhydrous tetrahydrofuran (50 ml) in a dry round bottom flask which had been flushed with nitrogen. A solution of 2.0 M 2-ethyl-3-methoxy-5-methylpyridine (6.9 ml) was added immediately and the mixture refluxed for 1 h. A deep red color slowly developed in the solution. It was then cooled and 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (1) was added as a fine powder until the color faded (800 mg). The mixture was stirred under reflux for a period of 20 min. Saturated ammonium chloride solution (20 ml) was added cautiously while the mixture was still under nitrogen. Ether (50 ml) was added to the reaction mixture and the organic phase washed with water before drying over sodium sulfate. Evaporation of the solvent *in vacuo* gave a pale yellow oil (1.1 g) which appeared primarily as one spot when examined by thin-layer chromatography; (canary yellow spot when the chromatoplate is developed with antimony pentachloride-carbon tetrachloride spray reagent). Column chromatography on alumina (55 g, activity III) eluting with 50% ligroin in benzene removed the unreacted pyridine (51). Continued elution with benzene removed the unreacted enone (1). The desired condensation product was eluted with 5% chloroform in benzene. Evaporation of the solvent afforded 23-methoxy-22,27-iminojerva-12(13),22,24,27-tetraene-3 β ,17-diol(s) as a pale yellow oil which crystallized from 5% chloroform in ether (700 mg), m.p. 209–214°. The spectral data indicated that the product is a mixture of two compounds of gross structure, 53; i.r. (chloroform): 3.05, 6.27 μ ; u.v.: λ_{max} (log ϵ) 283.5 nm (3.86); n.m.r.: 9.22 ($C_{19}CH_3$, 3H, singlet), 8.84, 8.81 ($C_{21}CH_3$, 3H, two overlapping doublets $J_{20,21} = 7.0$ Hz), 6.24, 6.21 ($-OCH_3$, 3H, two singlets), 3.10 ($C_{24}H$, 1H, singlet), 2.04 ($C_{27}H$, 1H, singlet); mass spectrometry: m/e 421 (M^+), prominent peaks at m/e 406 and 288.

Acetylation of 23-Methoxy-22,27-iminojerva-12(13),22,24,27-tetraene-3 β ,17-diols (53)

A mixture of acetic anhydride and pyridine (1:1, 5 ml) was added to the condensation product (53; 390 mg) obtained in the above experiment and left overnight at room temperature. The solution was then poured into crushed ice and the resultant oil was extracted with ether (3 $\times$ 20 ml). The combined ether extracts were washed with saturated sodium bicarbonate solution and several times with water before drying over sodium sulfate. The solvent was distilled off *in vacuo* when a pale yellow semisolid residue (330 mg) was obtained. Thin-layer chromatographic examination of the product revealed the presence of two compounds with very different R_f values. The less polar compound appeared as an orange-yellow spot when the chromatoplate was developed with antimony pentachloride reagent, while the second component appeared as a canary yellow spot. This mixture of acetates was separated by column chromatography on alumina (30 g, activity III). Elution with benzene afforded the less polar compound (designated as compound F) in essentially pure

state (125 mg). Further elution with 5% chloroform afforded the second component (designated as compound E, 191 mg).

An analytical sample of E was prepared by crystallization from 5% chloroform in ether whereupon colorless shining prisms were obtained, m.p. 181–182°; i.r.: 3.05, 5.84, 6.25, 6.39 μ ; u.v.: λ_{max} (log ϵ) 284 nm (3.86); n.m.r.: 9.22 ($C_{19}CH_3$, 3H, singlet), 8.82 ($C_{21}CH_3$, 3H, doublet, $J_{20,21} = 7.0$ Hz), 8.79 ($C_{18}CH_3$, 3H, singlet), 8.0 ($OCOCH_3$, 3H, singlet), 7.7 ($C_{26}CH_3$, 3H, singlet), 6.22 (OCH_3 , 3H, singlet), 3.90 ($C_{17}OH$, 1H, broad multiplet, disappears on D_2O addition), 3.08 ($C_{24}H$, 1H, singlet), 2.0 ($C_{27}H$, 1H, singlet); mass spectrometry: m/e 463 ($M - 18$), prominent peaks at m/e 448, 330, 288, 255, 146.

Anal. Calcd. for $C_{30}H_{41}NO_3$: C, 74.84; H, 8.94. Found: C, 74.65; H, 8.97.

Compound F, on the other hand, could not be induced to crystallize. An analytical sample of this material was prepared by subliming a small amount of B at 170°/0.2 mm, when a colorless glassy solid was obtained; i.r.: 5.83, 6.27, 6.39 μ ; u.v.: λ_{max} (log ϵ) 283.5 nm (4.03); n.m.r.: 9.08 ($C_{19}CH_3$, 3H, singlet), 8.45 ($C_{21}CH_3$, 3H, doublet, $J_{20,21} = 7.0$ Hz), 8.0 ($OCOCH_3$, 3H, singlet), 7.7 ($C_{18}CH_3$ and $C_{26}CH_3$, 6H, singlet), 6.3 ($C_{23}OCH_3$, 3H, singlet), 3.05 ($C_{15}H$ and $C_{16}H$, 2H, AB quartet, $J_{15,16} = 7.0$ Hz), 3.16 ($C_{24}H$, 1H, singlet), 2.0 ($C_{27}H$, 1H, singlet); mass spectrometry: m/e 461 ($M - 20$), prominent peaks at m/e 461, 446, 432, 298, 150.

Anal. Calcd. for $C_{30}H_{39}NO_3$ (mol. wt. 461.293): C, 78.09; H, 8.45. Found (high resolution mass measurement, 461.291): C, 77.78; H, 8.47.

3 β -Acetoxy-23-methoxy-22,27-iminojerva-12(13),14(15),16(17),22,24,27-hexaene: Compound G (57)

Compound E (54; 150 g) was thoroughly ground with 10% palladized charcoal (37 mg) until a homogeneous powder was obtained. This powder was heated at 200° under nitrogen for 7 min and the resultant solid residue washed several times with chloroform. Thin-layer chromatography of the chloroform extract indicated that the product contained three major components and these were separated on preparative layer plates (20 $\times$ 20 cm, 0.4 mm, chloroform). One of these three components was shown to be 2-ethyl-3-methoxy-5-methylpyridine (51) by n.m.r. The second band on extraction yielded a light oil which crystallized from acetone-petroleum ether as needles, m.p. 167°. This compound was identified as 3 β -acetoxy-5 α -etiojerv-12(13)-en-17-one (1) by its u.v. spectrum and mixture m.p. with an authentic sample. The third component which appeared as a light orange yellow spot when the chromatoplate was developed with antimony pentachloride-carbon tetrachloride reagent was extracted to yield a light oil which could not be induced to crystallize. An analytical sample of this compound, designated as compound G was obtained as a clear glass after distillation at 170°/0.1 mm; i.r.: 5.84, 6.27, 6.39 μ ; u.v.: λ_{max} (log ϵ) 283.5 nm (4.04); n.m.r.: 9.07 ($C_{19}CH_3$, 3H, singlet), 8.4 ($C_{21}CH_3$, doublet, $J_{20,21} = 7.0$ Hz), 8.0 ($OCOCH_3$, 3H, singlet), 7.74 ($C_{18}CH_3$ and $C_{26}CH_3$, 6H, singlet), 6.3 (OCH_3 , 3H, singlet), 3.1 ($C_{15}H$ and $C_{16}H$, AB quartet, $J_{15,16} = 8.0$ Hz), 3.18 ($C_{24}H$, 1H, singlet), 2.02 ($C_{27}H$, 1H, singlet); mass spectrometry: m/e 461 (M^+), prominent peaks at m/e 461, 446, 432, 298, 150.

Anal. Calcd. for $C_{30}H_{39}O_3$ (mol. wt. 461.293): C,

78.09; H, 8.45. Found (high resolution mass measurement, 461.293): C, 77.80; H, 8.46.

5 α ,6-Dihydroveratramine (56)

A solution of compound F (45 mg) in 95% ethanol (10 ml) containing 2% 12 *N* hydrochloric acid was stirred with Adams' catalyst (PtO₂, 20 mg) at 20° for 18 h. The mixture was filtered to remove the catalyst which was carefully washed with additional ethanol (5 ml). The combined extracts were neutralized with sodium bicarbonate solution and then reduced in volume to 2 ml *in vacuo*. It was diluted with water and extracted with chloroform (3 $\times$ 10 ml) before drying over sodium sulfate. Thin-layer chromatography on silica gel G (6% methanol in chloroform) revealed the presence of three major compounds. The two less polar components had similar *R_f* values, while the third compound had an *R_f* value identical with that of 5 α ,6-dihydroveratramine. Besides these three compounds, there were several other compounds present in the product mixture in trace amounts. The three major components were separated by thick-layer chromatography on neutral alumina (20 $\times$ 20 cm, 0.4 mm, 3% methanol in chloroform, plates developed three times). Extraction of the bands followed by the usual work-up afforded the two less polar compounds as pale yellow oils. The third compound, with an *R_f* value corresponding to that of 5 α ,6-dihydroveratramine, was obtained crystalline from 5% methanol in chloroform as colorless fluffy crystals (8 mg), m.p. 198°. This material was characterized as 5 α ,6-dihydroveratramine; undepressed m.p. (197–198°) and identical i.r., n.m.r., and mass spectra with an authentic sample.

The two less polar components obtained (17 and 13 mg, respectively) were found to be very unstable and hence no detailed investigation of these were carried out. A preliminary examination of the n.m.r. spectra revealed that the pyridine ring was only partially reduced in both the compounds (signals at 3.8 and 4.2) while the 23-methoxyl group was intact (signal at 6.3).

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CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit D

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ISOLATION OF TERATOGENIC ALKALOIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography was used for both analytical and preparative separations of several steroidal alkaloids which occur in extracts of *Veratrum californicum*. The inclusion of 0.1% trifluoroacetic acid in the mobile phase improved the efficiency of the chromatography and the solubility of the compounds in aqueous acetonitrile. Nuclear magnetic resonance was used to assist the identification of the isolated steroidal alkaloids. The effect of the interaction of trifluoroacetic acid with the alkaloids could be clearly seen by changes in the chemical shifts in the nuclear magnetic resonance spectra.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been used for the separation of a wide range of chemical compounds. Although there have been many reports of the use of HPLC in the separation of steroids, there have been only four reports to date of the application of HPLC to the separation of steroidal alkaloids. The earliest attempt used normal-phase chromatography on Porasil A, which is a very inefficient, coarse (37–75 μm particle size) support by modern standards, to separate tomatidine, solasodine, veratramine and jervine (Fig. 1) related alkaloids [1]. Surprisingly good separations were obtained by using gradients from acetone–hexane (2:1) to 97% aqueous acetone

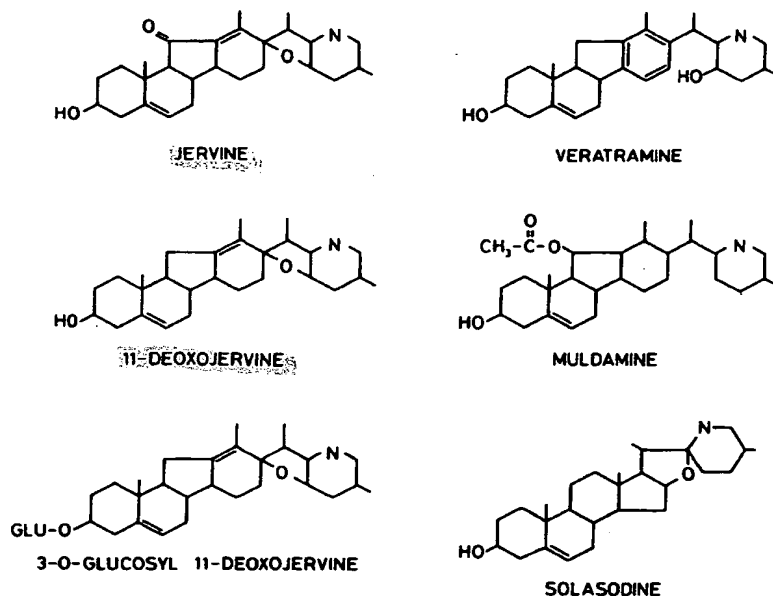


Fig. 1. Structural formulae of steroidal alkaloids.

and thin-layer chromatography as the mode of detection. Hunter et al. [1] used a 4.8 m long column of this material to prepare 1 g of tomatidine by isocratic elution. Normal-phase chromatography of these compounds was subsequently improved by Hunter et al. [2] by changing to a Zorbax-Sil (6 μ m particle size) support. The first reversed-phase HPLC separation was reported for the related glycoalkaloids, α -chaconine, β -chaconine and α -solanine [3]. The alkaloids were eluted isocratically from a C_{18} μ Bondapak column with tetrahydrofuran–water–acetonitrile (50:30:20). A more recent report of the separation of solasodine from solasodine glycosides also used a C_{18} μ Bondapak column, this time with a methanol–Tris buffer, pH 7 isocratic elution system [4].

We decided to develop a method for the purification of steroidal alkaloids from extracts of the roots of the plant *Veratrum californicum*. Crude extracts of the plant roots had been demonstrated to have teratogenic activity in a number of animals, the major defect being holoprosencephaly which sometimes results in cyclopia [5]. Hence the activity was referred to as "crude cyclopamine". Three alkaloidal steroids, jervine, 11-deoxojervine and glycoside of 11-deoxojervine, all found in the crude root extract, were found to have teratogenic activity [6]. We wish to obtain the main component, 11-deoxojervine in a pure form to study its mechanism of action [7].

EXPERIMENTAL

The chromatography equipment consisted of two Waters Assoc. Model 6000A solvent delivery systems controlled by a Model 660 solvent programmer and a Waters U6J injector. Peak detection was achieved by ultraviolet (UV)

absorbance at 254 nm using a 441 nm fixed-wavelength detector or at lower wavelengths with a 481-nm variable-wavelength detector (Waters Assoc.). Flow-rate was 1.0 ml/min throughout and all separations were performed at 22°C. The following columns were tested: three 30 cm × 3.9 mm C₁₈ μ Bondapak (10 μ m particle size) from Waters Associates, Carlton, Australia; two 25 cm × 4.6 mm ODS Ultrasphere (5 μ m particle size) from Beckman, Notting Hill, Australia; one 7.5 cm × 4.6 mm RPSC column (5 μ m particle size) from Beckman. Acetonitrile and methanol were both HPLC grade (Waters Assoc.), trifluoroacetic acid (TFA) was purchased from BDH and was purified prior to use by making up a 1% (v/v) aqueous solution and passing it through a C₁₈ Sep-Pak (Waters Assoc.) [8]. Glass-distilled water was further purified by a Milli-Q system (Millipore, Carlton, Australia) consisting of one charcoal, two ion-exchange and one Organics cartridge and a 0.45- μ m Millistack filter. Precise chromatographic conditions are given in the relevant figure legends and in the text. The measured pH of the mobile phase was between 1.79 for 20% acetonitrile–0.1% TFA to 1.92 for 60% acetonitrile–0.1% TFA. Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker 90 90-MHz spectrometer (Selby Scientific, Glen Waverley, Australia). Proton magnetic resonance data were obtained in the pulse/Fourier transform mode. NMR spectra were obtained at 300°K on steroidal alkaloids dissolved in C²HCl₃ containing tetramethylsilane (TMS) as internal standard. Sweep width was 1202 Hz, with either 4 K or 8 K real-data points collected to give a digital resolution of 0.34 or 0.17 Hz per point. Crude cyclopamine was prepared by benzene extraction of the roots of *Veratrum californicum* which were harvested in Idaho, U.S.A. The crude extract was partially purified by liquid partition through aqueous acid and chloroform and fractional crystallization as described previously [6, 9]. All other steroidal alkaloids were prepared as described previously [5, 6, 9].

RESULTS

Initial attempts to chromatograph veratramine, jervine and 11-deoxojervine by reversed-phase HPLC were hampered by two major problems, low solubility of 11-deoxojervine and jervine in water–methanol or water–acetonitrile or water–methanol–acetonitrile mixtures, and the peaks obtained for the three compounds on all three columns were asymmetric, with severe tailing. Crabbe and Fryer [4] had tried to overcome a similar problem using a methanol–Tris buffer, pH 7.0–7.5 mobile phase. This is a non-volatile solvent system, which still does not give efficient chromatography and narrow symmetrical peaks for the non-conjugated steroidal alkaloids. We found that a water–acetonitrile–0.1% TFA mobile phase solved both problems. All three alkaloids chromatographed on all three reversed-phase columns with greatly improved peak shapes. The C₁₈ μ Bondapak column proved to be the most efficient, typically showing 8000–10,000 plates per metre for jervine and 11-deoxojervine (Fig. 2). The ODS Ultrasphere columns were less efficient, (2000–3000 plates per metre) and the peak shapes for all three test compounds showed significant tailing. This result was found on two different ODS Ultrasphere columns and three different C₁₈ μ Bondapak columns. All columns were

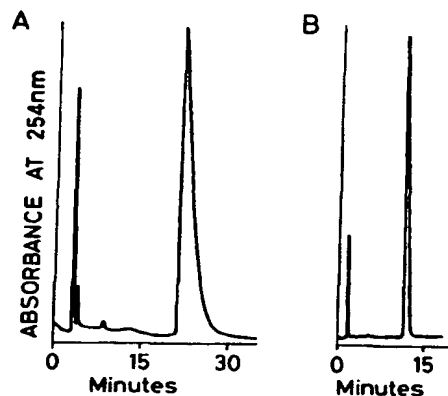


Fig. 2. Isocratic reversed-phase HPLC separation of jervine (A) without and (B) with 0.1% TFA in the mobile phase. A C_{18} μ Bondapak column was used for both chromatograms. (A) The mobile phase was acetonitrile-water (36:64); 20 μ g of jervine were injected in 50 μ l of the mobile phase; detector sensitivity was 0.05 a.u.f.s. (B) The mobile phase was acetonitrile-water-TFA (40:59.9:0.1); 20 μ g of jervine were injected in 50 μ l of the mobile phase; detector sensitivity was 0.1 a.u.f.s.

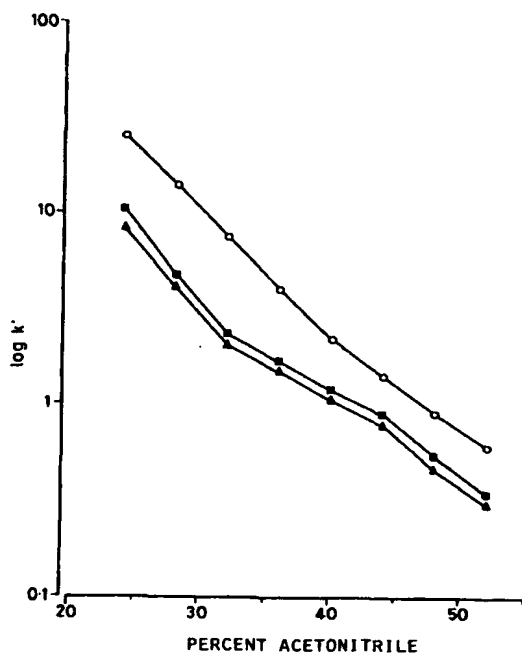


Fig. 3. Plot of $\log k'$ —acetonitrile concentration for jervine (▲), 11-deoxojervine (○) and veratramine (*). All data were obtained using the chromatographic conditions described for Figs. 4 and 5. TFA concentration was 0.1% throughout.

tested for efficiency using a standard uracil-acenaphthalene mixture, supplied with one of the C_{18} μ Bondapak columns by Waters Assoc. and an acetonitrile-water elution system (60:40, v/v, for the C_{18} μ Bondapak and 50:50, v/v, for the ODS Ultrasphere). Average efficiency for acenaphthalene on the C_{18} μ Bondapak columns was 16,500 plates per metre and on the ODS Ultrasphere columns was 33,800 plates per metre, under comparable conditions (flow-rate 1.0 ml/min, sample volume 10 μ l, k' between 3 and 6). Isocratic elution of the steroidal alkaloids with mixtures of between acetonitrile-water-TFA (28:71.9:0.1) and acetonitrile-water-TFA (40:59.9:0.1) gave good resolution of veratramine and jervine from 11-deoxojervine (Fig. 3). Although 11-deoxojervine was only sparingly soluble (<0.1 mg/ml) in acetonitrile-water-TFA (80:19.9:0.1) and in water-TFA (99.9:0.1), it was found to be much more soluble (5-10 mg/ml) in the range acetonitrile-water-TFA (20:79.9:0.1) to acetonitrile-water-TFA (65:34.9:0.1). The reason for this effect is probably due in part to the TFA forming an ion pair with the cyclic amine function.

Two different batches of crude cyclopamine were chromatographed isocratically in the acetonitrile-water-TFA system (Figs. 4 and 5). Although

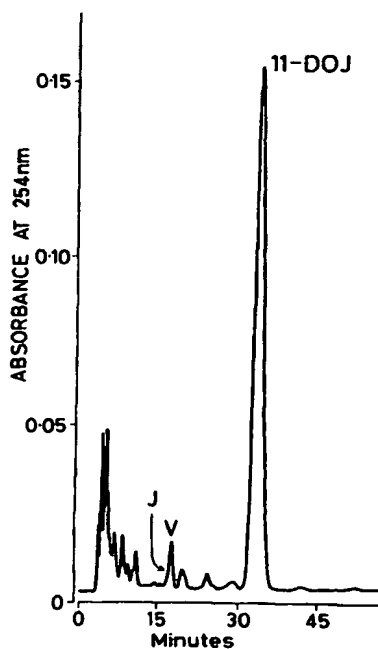


Fig. 4. Isocratic reversed-phase HPLC separation of crude cyclopamine "1981 batch". Crude cyclopamine (100 μ g) was injected in 50 μ l of the mobile phase, acetonitrile-water-TFA (32:67.9:0.1) onto a C_{18} μ Bondapak column. Detector sensitivity was 0.2 a.u.f.s. Peaks: J = jervine; V = veratramine; 11-DOJ = 11-deoxojervine.

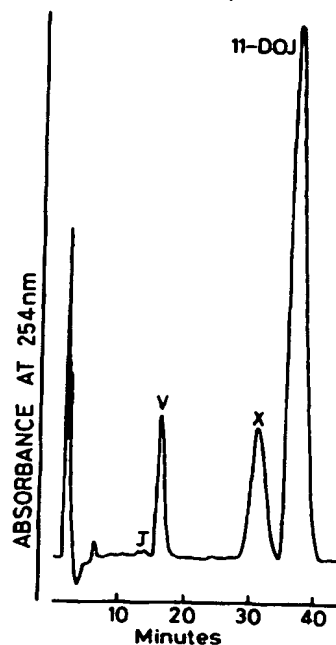


Fig. 5. Isocratic reversed-phase HPLC separation of crude cyclopamine "1982 batch". All conditions were as described for Fig. 4, except that the mobile phase was acetonitrile-water-TFA (28:71.9:0.1). Peaks: J = jervine; V = veratramine; X = unknown; 11-DOJ = 11-deoxojervine.

the major peak of the UV-absorbing material corresponded in elution position to 11-deoxojervine in both preparations, it was clear that the pattern of the minor peaks in the two extracts was different. Each pattern was always highly reproducible and there was no evidence for degradation. The reason for this is not clear, but it may be due to seasonal variation in the composition of the roots, as one extract was made twelve months before the other. The nature of the contaminant peak X (Fig. 5) is not known. It is important to note that different isocratic conditions were used for each extract. This was to produce the optimal resolution of the 11-deoxojervine from the various minor peaks. The glucoside of jervine was not retained on the column under either of the chromatographic conditions (Figs. 4 and 5). Muldamine and solasodine were both greatly retained on the column under these conditions ($k' > 100$) and were recovered by elution with 100% methanol or 100% acetonitrile. Large-scale (50–100 mg) preparations were successfully completed by trace enrichment of a dilute solution (1–2 mg/ml) of the crude cyclopamine in acetonitrile–water–TFA (20:79.9:0.1) onto a C_{18} μ Bondapak column, followed by isocratic elution at acetonitrile–water–TFA (32:67.9:0.1). Gradient elution of the steroidal alkaloids was also examined. An example is shown in Fig. 6 where 3-O-acetyl jervine, which was prepared from jervine by chemical synthesis [10], was eluted in a pure form by a acetonitrile gradient. One of the contaminants

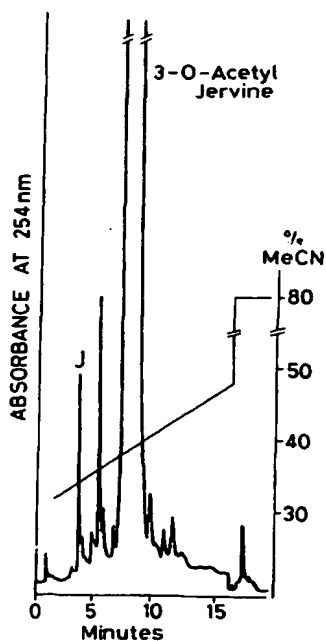
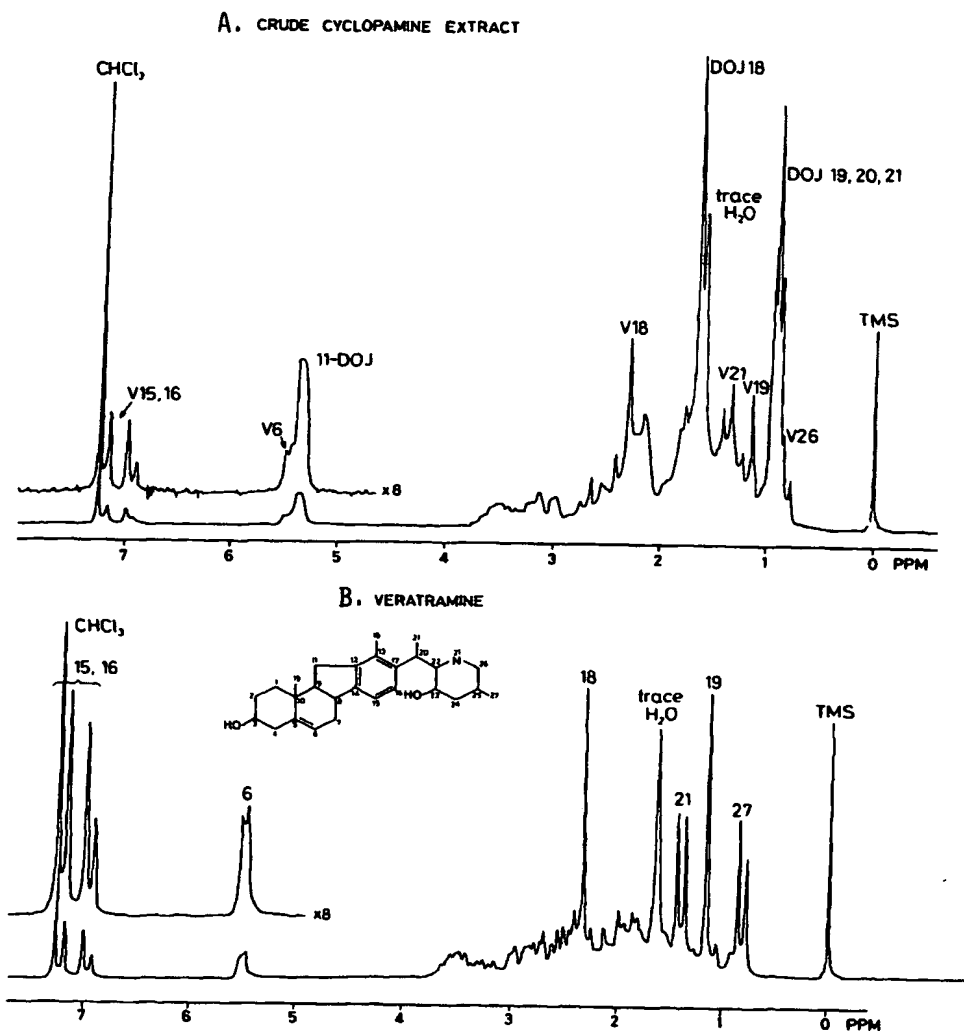


Fig. 6. Gradient chromatography of 3-O-acetyl jervine. A 450- μ g amount of 3-O-acetyl jervine was injected in 450 μ l of acetonitrile–water–TFA (32:67.9:0.1) onto a C_{18} μ Bondapak column equilibrated in the same mobile phase. The chromatogram was developed by a linear gradient from 32% to 48% acetonitrile containing 0.1% TFA throughout, over 15 min at a flow-rate of 1 ml/min. Detector sensitivity was 0.1 a.u.f.s. Peak: J = jervine.

was clearly free jervine. The identity of the 3-O-acetyl jervine was confirmed by NMR. Fig. 6 also demonstrates the efficiency of the chromatography at a moderate (450 μ g) sample loading. 3-O-Acetyl jervine is of importance as it represents the only radiolabelled form of jervine (3-O-[^{14} C]acetyl jervine) currently available.

In order to ensure that the material contained in the peaks obtained from the chromatograph was the desired compound, infrared and NMR spectra were run on the crude and purified alkaloids. The NMR spectra of crude cyclopamine, veratramine, and purified 11-deoxojervine are shown in Fig. 7A, B and C. The crude cyclopamine clearly had typical aromatic C—H resonances (Fig. 7A), around 7 ppm, which were similar to those seen in the NMR spectrum of



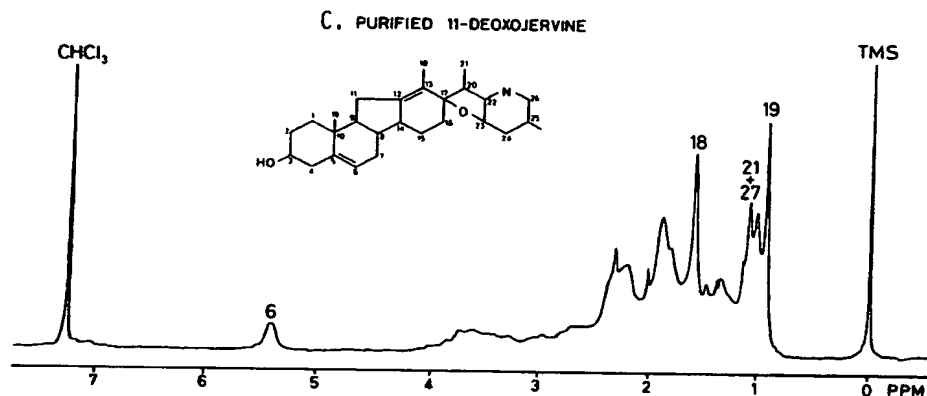


Fig. 7. Proton NMR spectra of (A) crude cyclopamine (B) veratramine and (C) reversed-phase HPLC purified 11-deoxojervine. Resonances in A which are marked with a V are due to contamination by veratramine or a closely related compound. The jervine numbering system of Brown [11] has been adopted here. Tentative assignments of the major resonances have been indicated.

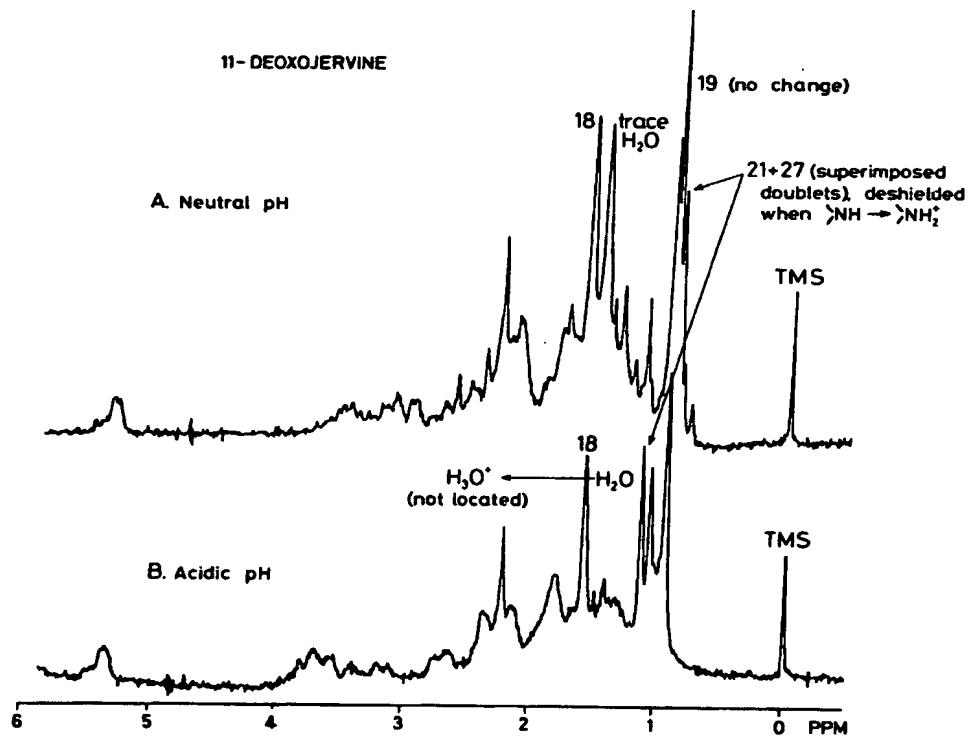


Fig. 8. Proton magnetic resonance spectra of crude cyclopamine (A) at neutral pH and (B) after acidification with 0.1% TFA. Note the pair of doublets at about 1 ppm which are deshielded by 0.176 ppm after acidification. The disappearance of the "trace H₂O" resonance in (A) is due to protonation of the H₂O, which results in a broader H₃O⁺ resonance at lower field.

veratramine (Fig. 7B). The purified 11-deoxojervine no longer had the aromatic resonances. The major resonances for the veratramine and 11-deoxojervine NMR spectra were tentatively assigned from the chemical shifts and *J* constants. The jervine numbering system of Brown [11] has been used. There was an obvious loss of the veratramine resonances (labelled V) when the crude cyclopamine was purified to yield pure 11-deoxojervine (Fig. 7). Another change was noted in the 11-deoxojervine spectra at around 1 ppm (see Fig. 7A and C). The pair of doublet resonances owing to the C₂₁ and C₂₇ methyl groups had shifted downfield in the purified 11-deoxojervine compared to the crude cyclopamine. This change could be easily explained when the NMR spectra of cyclopamine before and after acidification with TFA were examined (Fig. 8). Clearly acidification had caused the shift in C₂₁ and C₂₇ methyl resonances. We also examined the NMR spectra of jervine, 3-O-acetyl jervine, solasodine and muldamine [12] to assist with the assignments shown in Figs. 7 and 8.

DISCUSSION

We have successfully used reversed-phase HPLC to separate steroidal alkaloids with reasonable efficiency. The inclusion of 0.1% TFA in the mobile phase gave improved peak shapes and excellent recovery of material. We now routinely use isocratic reversed-phase HPLC (Figs. 4 and 5) to prepare pure 11-deoxojervine for our biological experiments, although it is clear that gradient chromatography can also be useful in the isolation of jervine and jervine derivatives (Fig. 6). One extremely useful result of our approach has been to dramatically improve the solubility of the crude cyclopamine. Traditionally this has always been a problem in any attempt to use crude cyclopamine in aqueous physiological environment. It has routinely been administered to experimental animals as a suspension with a solid carrier such as carboxymethyl cellulose [13]. After purification of the 11-deoxojervine by reversed-phase HPLC, we concluded that the basic nitrogen atom has a TFA anion complexed to it. This is supported by the chemical shift changes shown for the C₂₁ and C₂₇ methyl resonances (Fig. 8). We routinely exchange this TFA ion for chloride by diluting the eluate containing 11-deoxojervine 1:1 with 0.2% (v/v) hydrochloric acid and loading the purified 11-deoxojervine into a C₁₈ Sep-Pak [8]. After washing with 20 ml of aqueous 0.2% hydrochloric acid, we elute the alkaloid with 5 ml of 60% acetonitrile containing 0.2% (v/v) hydrochloric acid. This solution is diluted 1:1 with 0.2% (v/v) hydrochloric acid and is lyophilized. By this means, we obtain the pure 11-deoxojervine as a dry chloride salt which is then readily soluble in water (up to 2 mg/ml). Previous difficulties in solubility of the crude cyclopamine preparation must be put down in part to the presence of insoluble impurities, and in part to difficulty in forming the salt. In our hands, prior treatment with 0.1% TFA increased the solubility of 11-deoxojervine in 0.2% hydrochloric acid.

NMR spectroscopy was a very useful tool for the identification of the eluted alkaloids. As a general rule in HPLC, an alternative method of peak identification, other than elution volume, is mandatory. In the absence of a specific biological or immunological assay, spectroscopy seemed a reasonable

approach. Infrared spectra were generally found to be of a limited use (data not shown) although the presence of the carbonyl group in jervine gave a peak in the infrared spectrum at 1700 cm^{-1} which clearly distinguished jervine from 11-deoxojervine. We were able to obtain recognisable NMR spectra with amounts as low as 1.5 mg of steroidal alkaloid, although the spectra were much improved if 10 mg were used. The availability of a more powerful spectrometer (300 MHz) will improve the sensitivity of "detection" of the steroidal alkaloids eluted from reversed-phase HPLC by at least five-fold.

It can be seen that complete resolution was obtained between jervine and 11-deoxojervine, but that rather incomplete resolution could be obtained between jervine and veratramine (Figs. 3-5). We have found that a methanol-water-TFA mobile phase in the range 40-50% methanol will give complete resolution of jervine and veratramine [14] using isocratic elution and a C_{18} μ Bondapak column. The glucoside of jervine elutes just after the injection artefact in both the acetonitrile-water-TFA system and the methanol-water-TFA system. A ternary elution system comprising of aqueous TFA-methanol-acetonitrile may turn out to be the optimal isocratic system. Further studies will pursue this possibility.

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DECLARATION OF DR. SINAN TAS

Exhibit E

Kimonis VE et al, *American Journal of Medical Genetics* 1997;69:299-308.

Clinical Manifestations in 105 Persons With Nevroid Basal Cell Carcinoma Syndrome

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Nevroid basal cell carcinoma syndrome (NBCC; Gorlin syndrome), an autosomal dominant disorder linked to 9q22.3-q31, and caused by mutations in PTC, the human homologue of the *Drosophila patched* gene, comprises multiple basal cell carcinomas, keratocysts of the jaw, palmar/plantar pits, spine and rib anomalies and calcification of the falx cerebri. We reviewed the findings on 105 affected individuals examined at the NIH since 1985. The data included 48 males and 57 females ranging in age from 4 months to 87 years. Eighty percent of whites (71/90) and 38% (5/13) of African-Americans had at least one basal cell carcinoma (BCC), with the first tumor occurring at a mean age of 23 (median 20) years and 21 (median 20) years, respectively. Excluding individuals exposed to radiation therapy, the number of BCCs ranged from 1 to >1,000 (median 8) and 1 to 3 (median 2), respectively, in the 2 groups. Jaw cysts occurred in 78/105 (74%) with the first tumor occurring in 80% by the age of 20 years. The number of total jaw cysts ranged from 1 to 28 (median 3). Palmar pits and plantar pits were seen in 87%. Ovarian fibromas were diagnosed by ultrasound in 9/52 (17%) at a mean age of 30 years. Medulloblastoma occurred in 4 patients at a mean age of 2.3 years. Three patients had cleft lip or palate. Physical findings include "coarse face" in 54%, relative macrocephaly in 50%, hyper-

telorism in 42%, frontal bossing in 27%, pectus deformity in 13%, and Sprengel deformity in 11%. Important radiological signs included calcification of the falx cerebri in 65%, of the tentorium cerebelli in 20%, bridged sella in 68%, bifid ribs in 26%, hemivertebrae in 15%, fusion of the vertebral bodies in 10%, and flame shaped lucencies of the phalanges, metacarpal, and carpal bones of the hands in 30%. Several traits previously considered components of the syndrome (including short fourth metacarpal, scoliosis, cervical ribs and spina bifida occulta) were not found to be significantly increased in the affected individuals. This study delineates the frequency of the clinical and radiological anomalies in NBCC in a large population of US patients and discusses guidelines for diagnosis and management. *Am. J. Med. Genet.* 69:299-308, 1997.

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KEY WORDS: nevroid basal cell carcinoma syndrome (NBCC); basal cell carcinoma; keratocyst; pitting; ovarian fibroma; medulloblastoma; clinical features; radiological features; probands

INTRODUCTION

NBCC is an autosomal dominant disorder with an estimated prevalence of 1 in 57,000 [Farndon et al., 1992] to 1 in 164,000 [Shanley et al., 1994]. The gene has been mapped to 9q 22.3-q31 [Gailani et al., 1992; Farndon et al., 1992; Reis et al., 1992] with no evidence for heterogeneity [Chenevix-Trench et al., 1993; Wickling et al., 1994; Compton et al., 1994; Goldstein et al., 1994]. Recently, mutations in the human homologue of

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Drosophila patched, PTC, have been found to cause NBCC [Hahn et al., 1966; Johnson et al. 1996]. The gene is highly penetrant, but there is wide variation in the expressivity [Anderson et al., 1967]. The syndrome comprises multiple basal cell carcinomas, or early onset of BCC (i.e. one BCC prior to the age of 20 yr), keratocysts of the jaw, palmar/plantar pits, calcified dural folds, spine and rib anomalies, and characteristic face [Howell and Caro, 1958; Gorlin and Goltz, 1960]. The BCCs, which vary from a few to thousands, exhibit a wide range of appearance clinically and histopathologically, usually first appear around puberty and usually involve the face, back and chest. There is a difference in the frequency of BCCs between whites and African-Americans with NBCC [Goldstein et al., 1994]. Odontogenic keratocysts develop in more than 50% of NBCC patients often in the first decade. The cysts occur in both jaws and are lined by a thin layer of stratified squamous epithelium resulting in their designation as keratocysts. A well known trait is their recurrence estimated at 6 to 60% after enucleation of the keratocysts. Recurrence is partly attributed to the presence of satellite cysts [Dominguez and Keszler, 1988]. The palmar and plantar pits appear as shallow depressions which are due to partial or complete absence of the stratum corneum; they are found on the hands and feet, mainly on the palms and soles, but they can also be demonstrated on the sides, web spaces and dorsum of the fingers and toes [Howell and Mehregan, 1970].

On the basis of over 250 reports in the literature, Gorlin et al. [1963, 1965, 1971, 1972, 1987, 1995] identified several additional anomalies in the NBCC syndrome as listed in Tables I and V. Additionally radiological signs reported by several authors [Anderson et al., 1967; McEvoy and Gatzek, 1969; Dunnick et al., 1978] are listed in Table III. Individuals are prone to several specific neoplasms such as medulloblastoma [Herzberg and Wiskemann, 1963; Evans et al., 1991], other CNS tumors such as meningioma [Tamoney, 1969; Gorlin, 1987], ovarian fibroma which are often bilateral [Clendenning et al., 1963], and cardiac fibroma [Littler, 1979; Jones et al., 1986; Lacro and Jones, 1987; Coffin, 1992].

Two recent studies, one from the UK [Evans et al., 1993] and the other from Australia [Shanley et al., 1994], have reported the frequency of the manifestations in large series of NBCC patients. In our cross-sectional study of NBCC from the US population reported here we make comparisons between the frequency of the clinical and radiological signs in individuals with NBCC and their unaffected relatives to delineate the phenotype of NBCC.

METHODS

Patients were recruited to the National Institutes of Health for participation in a clinical and gene linkage study beginning in 1985. The study was approved by the Institutional Review Board and patient consent was obtained. For purposes of recruitment for this study, criteria used for diagnosis of NBCC syndrome were the presence of 2 major, or one major and 2 minor

criteria. The major criteria used included multiple BCCs or one BCC before 20 years, keratocysts of the jaw, palmar/plantar pits, lamellar calcification of the falx cerebri on skull radiograph, rib anomalies (bifid, synostosed, hypoplastic), ovarian fibroma, medulloblastoma, flame shaped lucencies in the phalanges, and brachymetacarpaly in the 4 limbs. Minor criteria included spina bifida occulta or other vertebral anomalies, brachymetacarpaly in at least one limb, hypertelorism or telecanthus, and frontal bossing. A diagnosis was also established by the presence of a first degree relative with NBCC and one major or two minor criteria.

We evaluated 20 multiple case families and 6 cases in which family history was negative. We also obtained clinical information on 105 individuals who fulfilled the criteria for NBCC and on 73 unaffected first degree relatives and 28 spouses. Patients underwent physical, dermatological, dental, and radiological examinations. Medical and family history were also obtained. Scoring of palmar and plantar pits was done on a scale of 0 to 3 (none, few, moderate, many). Dental evaluation included a panorex film. Radiological studies included anteroposterior (AP) chest, rib, spine, AP and lateral skull, hand and foot, long bones, and pelvis films. CT or MRI studies of the brain were obtained on 42 affected individuals (37 CT, 5 MRI) and 2 unaffected relatives had MRI studies. Ultrasound exam for ovarian fibromas was performed in 52 affected females and 37 unaffected females. All radiographs were reviewed by a single radiologist (BP).

Macrocephaly relative to height was determined by the formula of Bale et al. [1991] and the presence of telecanthus and hypertelorism by the method of Feingold and Bossert [1974].

Statistical Analysis

Statistical analysis was performed using SPSS 6.1 for Windows (SPSS Inc., Chicago, Illinois). Cross-tabulation analysis of the relationship between the recorded categorical variables (such as absence or presence of the various clinical features) by groups was performed using the Crosstabs procedure. Statistical differences between the observed and expected values in each group were tested using the Pearson chi-square test.

Comparison of the means of height, head circumference and eye measurements in the 3 groups was done using the analysis of variance (ANOVA) procedure.

The distribution of time to occurrence of the first BCC or jaw cyst in the affected individuals was performed using Kaplan-Meier Survival Analysis. Individuals who had not experienced an event (i.e. developed tumors) at the age of last observation or who did not survive long enough to develop tumors were censored. Statistical comparison between the whites and African-Americans was done using the Log-Rank test.

Imprinting

The effect of genomic imprinting, defined as differential expression of a gene depending on the sex of the parent who transmits it [Hall, 1990] was assessed. We compared the frequency of the various manifestations

of NBCC in affecteds who had inherited the disorder from their fathers ($N = 48$) versus those who had inherited it from their mothers ($N = 36$). Cross-tabulation analysis of the manifestations versus the parental transmission group was performed and statistical significance tested using the Pearson chi-square test.

Anticipation

The observation of increased severity of a disease phenotype in successive generations within the same family is known as anticipation [Sutherland and Richards, 1992]. We evaluated the data for the presence of anticipation as follows: Significant differences between generations was evaluated by performing a chi-square test of the frequency of structural anomalies in each generation. Each parent-child comparison was assessed independently. A parent with several children was included as many times as the number of children in the analysis. A total of 63 affected parent-child pairs was analyzed. The ages at first BCC or jaw cyst were not evaluated because of possible earlier ascertainment in younger generations.

RESULTS

The age range of the 105 affected persons was 4 months to 87 years (mean 34.5 yrs.). There were 48 males and 57 females (ratio 1:1.2) who fulfilled the criteria for NBCC. The 73 unaffected individuals ranged in age from 5.5 to 78 (mean 32) years. The male to female ratio of the unaffected individuals was 1:1.8. The status of 3 individuals (2 males and one female) was considered to be 'indeterminate' as they only partially met the diagnostic criteria for NBCC (i.e. first degree relative and one minor feature). Their ages were 5.2, 17 and 34 years. The spouses ranged in age from 20 to 87 (mean 47) years and their sex ratio was 1:1.5. Eighty-six percent (90/105) of the affected population was white, 13% (13/105) African-American and 2% (2/105) was Mexican/Asian.

SKIN FINDINGS

Basal Cell Carcinomas

Of the total population, 77 individuals had BCCs either by history or first noted at the time of their NIH evaluation. The age of first diagnosis of BCCs ranged from 3 to 53 years (mean 21.4 years, median 20 years). BCCs were seen mainly on the face but also on the trunk and limbs. The appearance of the BCCs ranged from small milia around the eyes and nose to large aggressive tumors. The data on BCCs in individuals with NBCC was analyzed separately for whites and African-Americans. In the whites, 80% (71/90) were noted to have one or more BCCs either at the time of examination or by previous report. The number of previous BCCs reported in whites ranged from 1 to >1,000 (mean 62, median 8). Most of the individuals who had developed BCCs reported multiple lesions by the time they were evaluated; the only affected individuals in whom a solitary BCC was documented were either African-American or under age 21 years. Among the Caucasians no correlation was found between the skin type

and the age of onset of first BCC or the total number of BCCs reported. Among the African-Americans, only 38% (5/13) developed one or more BCCs. (One of the African-American probands was a male who had medulloblastoma treated by radiotherapy at age 2 years [Korczak et al., 1997]. At the time of evaluation at 6.5 years he had approximately 100 BCCs in the radiation field.) In the remaining 4 African-Americans the total number of BCCs ranged from 1 to 3 with the first BCC occurring between the ages of 14 and 27 years. Our data included a 6.5 yr old Mexican-Asian male who had developed approximately 200 BCCs first diagnosed at age 6 years. His 44 year old father had moderate bilateral falx calcification and hemivertebra of the fourth thoracic vertebra, but no other clinical manifestation of NBCC.

Figure 1 shows the frequency of BCCs in whites at different ages with 97% over age 40 years having developed BCCs. A Kaplan-Meier lifetable analysis of the age at first BCC showed significantly different probabilities of developing BCCs at different ages in whites vs. African-Americans (Log-Rank test, $P = 0.001$). A probability curve of the risk of developing BCCs at specific ages is shown in Figure 2. Approximately 50% of affected whites developed their first BCC by the age of 21.5 years and 90% by 35 years. By contrast, in African-Americans there was approximately a 20% and 40% probability of developing BCCs at these ages, respectively.

One BCC was also found on examination of, or reported previously by, 2/73 unaffected individuals at ages 44 and 55 years. These individuals did not fulfill the criteria for diagnosis of NBCC as they had no other signs of NBCC and did not develop their BCCs prior to 20 years of age. One female developed two BCCs at age 65 years. This individual did not have any other findings of NBCC and subsequent genetic linkage studies confirmed that she had not inherited the gene for NBCC.

Radiation therapy for medulloblastoma administered in 3 individuals was associated with early development of BCCs in the radiation fields. These persons in whom BCCs occurred at ages 6, 9 and 12 years, developed BCCs significantly earlier than the overall

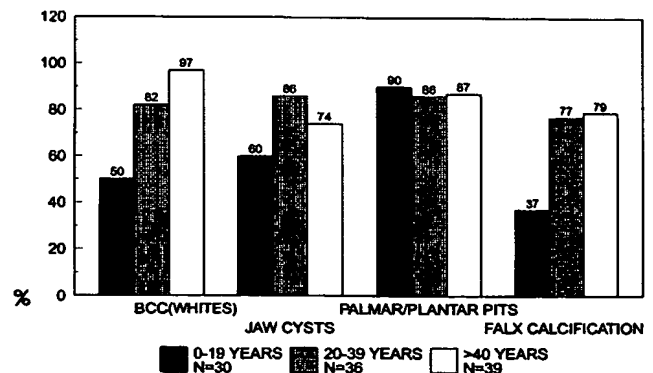


Fig. 1. Frequency of the major clinical signs: BCCs, jaw cysts, palmar/plantar pits and calcification of the falx cerebri in the different age groups: 0–19 years, 20–39 years and ≥ 40 years.

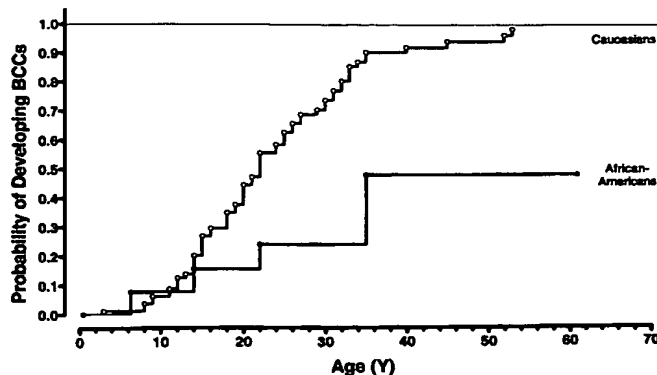


Fig. 2. A probability curve of the risk of developing BCCs at different ages in NBCC, in Caucasians versus African-Americans.

mean of 21.3 years in the entire sample ($P = 0.03$). One of these individuals developed BCCs which invaded the dura and caused his death at the age of 45 years. A 6.5-year-old African-American male had approximately 100 BCCs in the radiation field when examined at the NIH at the age of 6.5 years. The third case was a 26-year-old male who developed hundreds of BCCs over his back in the radiation field at the age of 12 years. Two individuals who received radiation for facial BCCs developed aggressive BCCs in the radiation fields. One female who received radiation for a 'birthmark' on the perineum in infancy was noted to have perineal BCCs at the age of 29 years.

Palmar and Plantar Pits

Pits were seen in 87% (89/102) of affected individuals in the palms and/or the soles. Palmar pits occurred in 86% (88/102), and plantar pits in 81% (79/97). Seventy-seven percent had palmar and plantar pits. Pitting of the palms was seen at approximately the same frequency in all age groups (Fig. 1). The youngest individual in whom pits were observed was 5 months old. Approximately 20% of children under the age of 10 years did not have visible pits on examination. Of individuals with pits, 51% had few pits, 28% had a moderate number of pits and 20% had many pits. Pitting was not observed in 13/102 (13%); these individuals ranged in age from 4 months to 61 years.

One unaffected individual was noted to have a single pit on a palm, and one spouse was noted to have two faint palmar pits. No unaffected individual was noted to have >3 pits.

JAW CYSTS

Seventy-eight of 105 (74%) individuals with NBCC had a history of jaw cysts or a jaw cyst detected on dental evaluation at the NIH. The age of onset of the jaw cysts ranged from 4 to 67 years (mean 17.1 years, median 13 years). The number of jaw cysts ranged from 1 to 28 (mean 5.1, median 4.5). Five individuals had more than 10 jaw cysts in their lifetime. The Kaplan-Meier life table analysis showed that the onset of jaw cysts correlated with the age of the patient; however, 75% developed jaw cysts by the age of 20 years (Fig. 3).

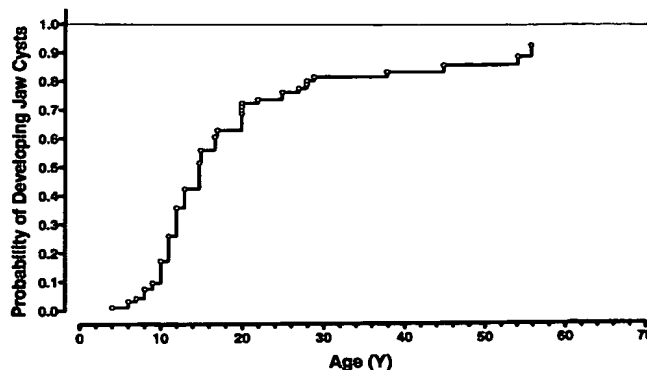


Fig. 3. A probability curve of the risk of developing jaw cysts at different ages among all patients with NBCC. There was no difference between African-Americans and Caucasians.

One affected individual was found to have an ameloblastoma. No unaffected individual or spouse had jaw cysts.

MEDULLOBLASTOMAS

Four individuals had a medulloblastoma diagnosed between the ages of 2 and 3 years. Radiotherapy for the medulloblastoma had a marked effect on the early development of BCCs and other cerebral tumors. One girl died at 5 years from increased intracranial pressure due to secondary cerebellar ependymoma development. One of the 3 surviving individuals was a 25 year old man who, in addition to developing hundreds of BCCs in the radiation field, developed neurological symptoms at the age of 26 years. MRI demonstrated several meningiomas, one of which was compressing his brain stem.

GENITOURINARY ANOMALIES

Fifty-two of the women and girls with NBCC were evaluated by pelvic ultrasound examination; 9 were found to have ovarian fibromas (17%). Their age ranged from 16 to 45 years (mean 30.6 years). Of women over the age of 30 years, 5/27 (18.5%) had ovarian fibromas detected by ultrasound. One woman with NBCC had a septate uterus. In none of the 37 unaffecteds who had pelvic ultrasound evaluation was an ovarian fibroma detected.

Among males, one affected and one unaffected (unrelated) individual had hypospadias. Four affected and one unaffected male had a history of cryptorchidism at birth. Three of these cases occurred in the same family—a father and son who both had NBCC and an unaffected son. All 3 had unilateral cryptorchidism requiring surgery.

PHYSICAL CHARACTERISTICS

Table I presents the physical findings for the 3 groups of relatives. Many cranio-facial anomalies were significantly more prevalent among the persons with NBCC. A metacarpal sign, previously associated with NBCC, was not found to be significantly increased in

TABLE 1. Frequency of Clinical Signs in Affected Individuals With NBCC Syndrome, Their Unaffected Relatives and Spouses

	Affected (%)	Unaffected (%)	Spouses (%)	P
Craniofacial anomalies				
Frontal bossing	27/103 (26)	0/68 (0)	1/25 (4)	<0.01
Skull shape abnormalities ^a	16/102 (16)	3/68 (4)	0/25 (0)	0.05
Macrocephaly-males >20 yr. ^b	15/28 (54)	2/16 (13)	1/11 (9)	0.01
Macrocephaly-females >20 yr. ^b	18/39 (46)	3/28 (11)	3/14 (21)	0.01
"Coarse face"	53/101 (52)	1/68 (2)	0/24 (0)	<0.01
Epicanthic folds	17/102 (17)	9/68 (13)	2/24 (8)	0.54
Synophrys	35/102 (34)	12/68 (17)	2/24 (8)	<0.01
Ear pits	6/70 (9)	2/49 (4)	0/23 (0)	0.25
Eye anomalies				
Strabismus	19/101 (19)	1/68 (2)	1/25 (4)	<0.01
Hypertelorism ^c	41/98 (42)	6/49 (12)	1/25 (4)	<0.01
Telecanthus ^c	25/97 (26)	6/49 (12)	2/25 (8)	0.04
Oropharyngeal anomalies				
Cleft palate	3/105 (3)	0/73 (0)	0/25 (0)	<0.01
Other palate abnormalities ^d	47/102 (46)	11/66 (20)	0/23 (0)	<0.01
Skeletal anomalies				
Pectus deformities	12/98 (12)	0/58 (0)	0/15 (0)	0.02
Sprengel deformity	11/101 (11)	0/61 (0)	0/16 (0)	0.03
Metacarpal sign ^e	10/99 (10)	4/69 (6)	0/23 (0)	0.19
Syndactyly	24/102 (24)	3/65 (5)	1/22 (5)	0.19

^aDolichocephaly and brachycephaly.^bBale et al., 1991.^cFeingold and Bossert, 1974.^dHigh-arched palate or prominent palatine ridges.^eShort 4th metacarpal- unilateral/bilateral.

the affected group. Ten percent of affected and 6% of unaffected persons, but none of the spouses, had definite unilateral or bilateral short fourth metacarpals.

Physical Measurements

Mean values of the physical measurements in the 3 groups were analyzed in individuals over the age of 18 years. Males and females were considered separately (Table II). The mean height and lower body segment were significantly increased in the affected group compared to the unaffected or spouse group. The upper/lower body segment ratio was normal in the affected individuals indicating that they were proportionately taller individuals rather than having disproportionately long limbs. Using the formula of Bale et al. [1991], the head circumference 2 standard deviations above the mean (95th centile) was calculated in adults over the age of 20 years standardized to gender and height. Relative macrocephaly was present if the observed head circumference exceeded the predicted head circumference. Relative macrocephaly was observed at a higher frequency in affected (50%) compared to unaffected individuals ($P = 0.01$) (Table I).

Eye measurements showed that 42% in the affected group were hypertelorism (interpupillary distance >2SD from the mean). Telecanthus (inner-canthal distance >2 SD from the mean), related to hypertelorism was seen more frequently in the affected population.

RADIOLOGICAL FINDINGS

Table III presents the radiological signs evaluated in the affected and unaffected persons.

Skull Radiographs

Calcification of the falx cerebri was observed in 65% of all affected individuals. Figure 1 shows that 23/29 (79%) individuals over the age of 40 years had falcial calcification, 20/26 (77%) between the ages of 20 and 40 years and 10/27 (37%) individuals under the age of 20 years. Faint calcification of the falx was seen in 2 unaffected males over 50 years. Neither had any other signs of NBCC. Calcification of the tentorium cerebelli and bridging of the sella turcica was also observed more frequently in the affected individuals.

Chest and Rib Radiographs

Thirty-eight percent of individuals with NBCC had rib anomalies which included bifid ribs, marked widening of the anterior ends of the ribs, fusion and modelling defects of the ribs. The third, fourth and fifth ribs were predominantly involved; however, abnormalities were occasionally seen in other ribs. There was no difference between the groups in the incidence of cervical and absent/rudimentary ribs.

Spine Radiographs

Scoliosis was observed more frequently in affected individuals; however, this difference was not significant. Several affected individuals however were noted to have more severe scoliosis, which was often at the site of developmental abnormalities such as hemivertebrae or fusion of the vertebral bodies. Spina bifida occulta was not observed more frequently in the NBCC population compared to the unaffected individuals.

TABLE II. Comparison of Means of Measurements in Individuals >18 Years*

Sex	Males				Females			
	Affected	Unaffected	Spouse	P	Affected	Unaffected	Spouse	P
Status	30	18	11		42	29	14	
Number of persons								
Height	180.5	175.6	173.1	0.03	172.4	164.8	162.7	<0.01
Head circumference	59.4	57.4	56	<0.01	57.8	55.2	55.2	<0.01
Middle finger length	8.7	8.1	7.8	<0.01	8.1	7.8	7.8	0.04
Hand length	21	20	19.6	<0.01	18.8	17.5	17.9	<0.01
Eye measurements								
Inner-canthal	3.6	3.2	3.3	<0.01	3.3	3.1	3	0.04
Inter-pupillary	6.9	6.3	6.3	0.02	6.4	6	6	<0.01
Outer-canthal	10	9.5	9.4	0.03	9.4	8.8	8.7	<0.01
Ratio upper/lower segment	0.9	1	1	0.3	0.9	0.9	0.9	0.77
Ratio middle finger/hand	0.4	0.4	0.4	0.75	0.4	0.4	0.4	0.23

*All measurements in cm.

Limbs

Flame shaped lucencies reported previously in NBCC [Dunnick, 1978] were seen on 24/80 (30%) hand films, and on 9/53 (17%) foot films. No flame shaped lucencies were observed in the unaffected persons. Polydactyly of the feet was observed in one child and was reported in one affected woman who had previous corrective surgery for this anomaly.

CT/MRI Studies

Abnormalities of the cerebrum were found in 18/42 (42.8%). Findings are reported in Table III. Falx and tentorial calcification was more easily visible on CT scans compared to skull films. In 2/10 persons with asymmetric or dilated ventricles, findings were secondary to neurosurgery and in the remaining individuals were related to communicating mild hydrocephalus. Cerebral atrophy was present in 10% and dysgenesis or agenesis of the corpus callosum in a further 10%. These individuals did not have any other detectable anatomical abnormalities of the cerebrum. The individual referred to above with medulloblastoma had neurological symptoms and signs attributable to multiple meningiomas as seen on MRI.

PROBANDS VERSUS NON-PROBANDS

The frequency of the various manifestations of NBCC in the probands ($n = 26$) was compared with non-probands with NBCC ($n = 79$). Anomalies found to be more prevalent among probands ($P = <0.05$) were the more visible changes of NBCC, for example, the frequency of BCCs (in whites only), pits, frontal bossing, macrocephaly, and pectus deformity. Other defects, in particular the frequency of jaw cysts, hypertelorism, Sprengel deformity, height and radiological signs associated with NBCC were not found to be significantly increased in the probands. BCCs were also observed more frequently among probands than non-probands (approximately 2:1) among African-Americans. However, this difference was not significant (data not shown).

INDETERMINATE CASES

Of the 3 cases in this group, two individuals were under the age of 20 years and had not developed the

clinical manifestations of NBCC. The youngest was a 5-year-old boy with a large head, "coarse face", bifid uvula and ptosis who had not developed other signs of NBCC. His 4-month-old brother had severe bilateral clefting of the lip and palate, macrocephaly and "coarse face" and his mother had both major and minor traits of NBCC. A 17-year-old woman who was shown to be a definite gene carrier by linkage analysis, had some facial characteristics and hypodontia with 13 congenitally absent teeth. However, she did not fulfill the criteria for the diagnosis of NBCC used in this study. A 33-year-old African-American man had a radiolucency in his jaw when evaluated at the NIH and no other definite findings of NBCC. This individual refused further medical follow-up.

FAMILY STUDIES

Considerable variation in the clinical expression of NBCC was observed within families. In the largest family evaluated with 18 affected individuals, 17 developed their first BCC at ages ranging from 8 to 35 years and 16 developed their first jaw cyst at ages ranging from 7 to 67 years. The frequency of other major signs (e.g., number of pits, presence of bifid ribs, and vertebral anomalies) also varied among relatives. Intra-familial variation was difficult to ascertain because of the enormous intra-familial variation and the small size of many families. Statistical comparisons were made between the two largest families comprising more than 10 affected relatives. Syndactyly of the second and third toes was found to cosegregate with NBCC in one large family. Fourteen of the 18 affected individuals in this large family had syndactyly compared to 1/19 unaffected members ($P < 0.01$). By contrast, in the other large family, only 1 of 12 affected and none of the 11 unaffected relatives had syndactyly of the second and third toes. Other defects that were significantly different ($P < 0.05$) in these two families were the frequencies of strabismus and bridging of the sella. All other clinical and radiological anomalies were prevalent at similar frequencies.

The effect of genomic imprinting assessed by comparing the frequency of clinical signs of NBCC in affected individuals who had inherited the gene from their fathers versus those who had inherited it from their mothers showed that there were no significant

TABLE III. Radiological Findings in Affected Persons vs. Unaffected Relatives

	Affected N (%)	Unaffected N (%)	P
Chest and ribs			
Bifid ribs	21/82 (26)	0/32 (0)	<0.01
Splayed/fused ribs	13/82 (16)	0/32 (0)	<0.01
Cervical ribs	3/80 (4)	2/36 (6)	0.65
Absent/rudimentary ribs	5/82 (6)	2/32 (6)	0.13
Skull			
Falx calcification	53/82 (65)	2/38 (5)	<0.01
Tentorium cerebellum calcification	16/82 (20)	0/35 (0)	<0.01
Bridged sella	57/84 (68)	9/35 (26)	<0.01
Spine			
Scoliosis	23/75 (31)	5/33 (15)	0.09
Hemivertebrae	11/74 (15)	0/31 (0)	0.02
Fusion of the vertebral bodies	7/73 (10)	0/30 (0)	0.08
Spina bifida occulta	14/75 (19)	4/32 (13)	0.44
Hands and feet			
Flame shaped lucencies-hand	24/80 (30)	0/28 (0)	<0.01
Flame shaped lucencies-feet	9/53 (17)	0/8 (0)	0.2
Brain CT or MRI			
Asymmetric ventricles	10/42 (24)	0/2 (0)	ND*
Cerebral atrophy	4/42 (10)	0/2 (0)	ND
Dysgenesis/agenesis corpus callosum	4/42 (10)	0/2 (0)	ND
Meningioma	2/42 (5)	0/2 (0)	ND

*ND = P values not calculated.

differences in the frequency of clinical anomalies in the 2 groups. Frontal bossing was seen more frequently among individuals who had inherited NBCC from their mothers (14/36) compared to those who had inherited NBCC from their fathers (7/48) ($P = 0.02$). Other neoplasms, in particular jaw cysts, ovarian fibromas or medulloblastomas, also were not more frequent in one group.

Anticipation, assessed by comparing the frequency of manifestations in the parent generation with the child generation, indicated that there was a higher frequency of palatal abnormalities, such as high arched palate, among the offspring group (28/60) compared to the parent group (12/60) ($P = 0.01$). Cleft palate was observed in 3 offspring. There was an increased frequency of Sprengel deformity in the offspring group (9/62) compared to the parent group (3/59) but this difference was not statistically significant ($P = 0.08$). No significant differences in the two groups in the incidence of other physical or radiological traits of NBCC was observed.

COMPARISON WITH OTHER LARGE STUDIES (TABLE IV)

The diagnostic criteria used by Shanley et al. [1994] were modifications of those used by Chenevix-Trench et al. [1993]. In Evans' study [1993] similar major but fewer minor criteria were used. Given the extreme variation in expression of NBCC, and the different diagnostic criteria used, the 3 studies are remarkably similar in the distribution of the major and minor characteristics. The frequency of BCCs in the UK population over the age of 20 years was lower, but this difference was not significant ($P = 0.05$). More frequent palmar/plantar pitting in the NIH population was found compared to the UK group ($P = 0.01$); however, differences were not significant when compared with the

Australian study. More frequent falx calcification in the Australian study was reported compared to the frequency in the NIH study ($P = <0.01$). Frequency of falx calcification was not reported in the UK study.

DISCUSSION

We have examined the clinical and radiological data of 105 persons with NBCC. Characteristics seen in more than 50% of individuals with NBCC are pits, BCCs, jaw cysts and falx calcification; these anomalies are included as major criteria. At least 2 of these major criteria of NBCC were found in 100/105 persons in our study. The remaining 5/105 individuals ranged in ages from 9 months to 21.5 years. Bifid ribs were seen in 3 of these individuals and the presence of 2 or more minor signs of NBCC (hemivertebrae in 1, macrocephaly in 2, hypertelorism in 2, telecanthus and frontal bossing in 1) helped in establishing the diagnosis in the remaining two individuals at age 6 and 9 years.

Diagnostic criteria used initially for recruitment can be modified based on the results of our study. Our suggested criteria for the diagnosis of NBCC is listed in Table V. Bifid/splayed/synostosed ribs, which were seen in 42% of affected individuals, may be considered a major criterion of great help in establishing a diagnosis particularly in the pediatric population. The frequency of bifid ribs in the general population is very low, having been reported as 3 to 6.2 per 1,000 [Etter, 1994; Strong, 1977].

The increased frequency of BCCs in whites (80%) compared to African-Americans (38%) has been reported in other studies [Anderson et al., 1967; Goldstein et al., 1994]. The lower frequency of BCCs in the African-American group may be related to increased protection from ultraviolet light because of the skin pigmentation. The role of sun exposure in the development of BCCs in white persons with NBCC was evalu-

TABLE IV. Comparison of Anomalies of NBCC Among Three Studies

	Evans et al. (United Kingdom)	Shanley et al. (Australia)	NIH study (United States)
Number of cases	84	118	105
Number of families	29	64	26
Mean age (Y)		35	34.5
Sex ratio M:F	1:1.3	1:1.3	1:1.2
Number with BCCs-total (%)	33/70 (47)	90/118 (76)	71/90 (80) ^a
Age >20 Y (%)	33/45 (73)	71/84 (85)	58/64 (91) ^a
Age >40 Y (%)	19/21 (90)	35/37 (95)	34/35 (97) ^a
Mean age first BCC (Y)		20.3	21.4
Number with jaw cysts-total (%)	46/70 (66)	85/113 (75)	78/105 (74)
Age >20 Y (%)	37/45 (82)	66/82 (80)	60/74 (81)
Age >40 Y (%)	19/21 (90)	25/35 (71)	29/38 (76)
Range of total number of jaw cysts (mean)	NA	1-28 (6)	1-28 (5.1)
Mean age first jaw cyst (Y)	NA	15.5	17.3
Pitting-palms/soles (%)	50/70 (71)	82/103 (80)	89/102 (87)
Cleft lip/palate (%)	4/70 (5)	4/107 (4)	3/103 (3)
Calcification of falx cerebri (%)	NA	81/89 (92)	53/82 (65)
Medulloblastoma (%)	3/84 (4)	1/118 (1)	4/105 (4)
Ovarian fibromas (%)	6/25 (24)	9/63 (14)	9/52 (17)

^aRestricted to whites.

ated by Goldstein et al. [1993] by questionnaire. They reported that 88% of BCCs in women and 86% in men from the general population occurred in sun-exposed areas of the body versus 59% in women and 65% men with NBCC. They concluded that the anatomic site distribution suggested that sun may not be an essential factor in the development of BCCs. The increased frequency in the sun-exposed areas however, suggested that sun may exacerbate the development of BCCs. They reported no significant correlation between total number of BCCs and recall of hours of lifetime sun exposure.

Palmar pits were found to be the most prevalent sign of NBCC, seen at approximately the same frequency in all age groups, therefore representing a very useful diagnostic trait of NBCC even at an early age. The incidence of pits appeared to be higher in our study than

some others. The reason for this difference is unexplained but interfamilial variation is a possibility.

No medulloblastoma occurred after the age of 3 years in this study. Evans et al. [1991] reviewed the literature and reported an average age of diagnosis of 2.1 years in 20 cases, the ages at diagnosis ranging from 2 months to 7 years. Thus, it is very important to screen for medulloblastoma in the early years of life in patients at risk of NBCC. Our finding of early onset of BCCs in the radiation fields (mean latency period 5 yr. after exposure to radiation) was also reported by these authors. Dinehart et al. [1991] reported (3 young patients) (2 with acute lymphoblastic leukemia, and 1 with astrocytoma) who developed a BCC of the scalp 8-15 years after irradiation. The multiple meningiomas we observed in one individual (latency period 22 yr.) and the cerebellar ependymoma in another (latency period 2 yr.) was likely related to the radiation therapy for medulloblastoma. Radiation-induced second intracranial neoplasms, in particular multiple meningiomas, and ependymomas have been reported in other studies [Iacono et al., 1981; Anderson and Treip, 1984; Moss et al., 1988; Mack and Wilson, 1993]. The average latency period from radiation therapy to diagnosis of a meningioma was 24 ± 10 yr in the general population [Mack and Wilson, 1993].

The lower incidence of macrocephaly in our population compared to that of Shanley et al. [1994] may have occurred because we adjusted for height in our study. NBCC was previously shown to be associated with macrocephaly in probands while non-probands were not found to have significantly greater head circumferences than predicted [Bale et al., 1991]. Analysis of our data which included the 40 individuals reported by Bale et al. and an additional 27 persons with NBCC over the age of 20 yr. also showed that probands had a higher frequency of macrocephaly than other affected individuals in the families ($P < 0.01$).

The most frequent radiological sign of NBCC among affected individuals at all ages was falx calcification

TABLE V. Diagnostic Criteria for NBCC Syndrome

Diagnosis of NBCC made in the presence of two major or one major and two minor criteria:

Major criteria

1. More than 2 BCCs or one under the age of 20 years
2. Odontogenic keratocysts of the jaw proven by histology
3. Three or more palmar or plantar pits
4. Bilamellar calcification of the falx cerebri
5. Bifid, fused or markedly splayed ribs
6. First degree relative with NBCC syndrome

Minor criteria

Any one of the following features:

1. Macrocephaly determined after adjustment for height
2. Congenital malformations: cleft lip or palate, frontal bossing, "coarse face," moderate or severe hypertelorism
3. Other skeletal abnormalities: Sprengel deformity, marked pectus deformity, marked syndactyly of the digits
4. Radiological abnormalities: Bridging of the sella turcica, vertebral anomalies such as hemivertebrae, fusion or elongation of the vertebral bodies, modeling defects of the hands and feet, or flame shaped lucencies of the hands or feet
5. Ovarian fibroma
6. Medulloblastoma

(65%), the incidence increasing with age. Dunnick et al. [1978] found calcification of the falx in 85% individuals studied radiologically, and Shanley et al. [1994] reported an incidence of 92%. A bridged sella was seen in 68% of affected individuals, but also in 26% of unaffected relatives and, therefore was not found to be a discriminating sign for NBCC. Its frequency in the general population is reported to be 7% [Heublain, 1946]. In the current study, rib anomalies were seen in 43% of the affecteds which is comparable to other studies [Shanley et al., 1994; Dunnick et al., 1978]. The finding of increased frequency of developmental abnormalities involving ribs 3 to 5 in this study is interesting. Vertebral anomalies such as hemivertebrae and fusion of the vertebral bodies were seen in 31% of affected persons similar to the report by Dunnick et al. [1978]. Flame shaped lucencies of the hands were seen in 30% in our study and in 46% in the Dunnick study. The incidence of polydactyly (3%) was similar to that obtained by Shanley et al. [1994].

Absent or abnormal corpus callosum was seen as an autopsy finding in the first reported case of NBCC [Binkley and Johnson, 1951]. Other associated anomalies such as asymmetric ventricles (not associated with tumors or surgery) and cerebral atrophy have not previously been reported. Falx and tentorial calcification was seen at an earlier age on CT evaluation of the cerebrum compared to X-rays. However, in view of the exposure to radiation resulting from CT examination this investigation is no longer routinely done. MRI studies are recommended instead for detection of medulloblastomas in children.

On comparing the age of first BCC, age of first jaw cyst and frequency of anomalies in several generations of our large multi-generational families we found no evidence of anticipation. Similarly the incidence of anomalies in individuals who had inherited NBCC either from their mother or father was compared and not found to be statistically different (except for a higher frequency of frontal bossing in offspring of affected mothers). These findings contrast with the results of Shanley et al. [1994]. We found extensive intra-familial and inter-familial clinical variability which makes this type of analysis extremely difficult.

Anomalies previously reported to be associated with NBCC such as a metacarpal sign, hypospadias, cryptorchidism, anosmia and radiological features such as spina bifida occulta, and scoliosis [Gorlin, 1987] were not found to be significantly increased in affected persons compared to the two control groups in our study. For example, a metacarpal sign was seen in 10% affected and 6% of unaffected persons, these rates being close to the reported rate of 10% metacarpal sign in the general population [Bloom, 1970].

In summary, our study of 105 affected individuals and their unaffected relatives indicated that the frequency of the findings of NBCC in the US population was very similar to those obtained in other population groups (British and Australian) [Evans et al., 1993; Shanley et al., 1994]. Children proved the greatest challenge in making a diagnosis of NBCC as several of the signs of the syndrome develop over time, in particular the jaw cysts, BCCs, calcification of the falx

cerebri and ovarian fibromas. Evaluation for palmar and plantar pits and radiological anomalies such as bifid ribs may prove useful indicators of NBCC.

RECOMMENDATIONS

A child who is at risk for having inherited the gene for NBCC should have careful physical evaluation at birth for pits and minor features of NBCC and radiological evaluation with rib, skull, and spine films to look for skeletal abnormalities such as bifid, fused or accessory ribs, hemivertebrae or fused vertebrae. The risk of developing a medulloblastoma in our study is greatest between 2–3 years; however has occurred up to age 7 yrs [Evans et al., 1991]. Therefore, careful regular neurological surveillance at 6 month intervals in addition to annual MRI of the cerebrum is strongly recommended up to age 7 years. Early detection would facilitate more complete surgical eradication of the tumor. In our study we do not report any cases of cardiac fibromas. (Evans et al. [1993] report an incidence of 3% of cardiac fibroma in NBCC.) Therefore echocardiographic evaluation is recommended at birth for a baby at risk and subsequently on clinical suspicion. Although most individuals develop their first BCC in their early 20s, children as young as 1.5 years in the absence of radiation therapy have been noted to have BCCs. Therefore regular dermatological surveillance from an early age with the frequency of visits increasing as needed during and after adolescence is important. Advising patients to reduce exposure to ultraviolet radiation may lessen their risk of developing BCCs. Radiation therapy should be avoided in NBCC if at all possible because BCCs are much more frequent and aggressive in the radiation fields and the risk of other secondary tumors is also increased. Since keratocysts of the jaw can develop as early as 4 years, regular early dental surveillance with annual panorex films is recommended as soon as the child can comply with the examination procedure. (Complete removal of the cysts is essential because of the high rate of recurrence.) Ovarian fibromas generally develop in the teen years; however they have been reported as early as 3.5 years. Most of these ovarian fibromas are asymptomatic and almost never become malignant. Initial ultrasound evaluation at the pre-teen stage is recommended, and repeated examination should occur if the individual becomes symptomatic.

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Exhibit F

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Mutations of the *PATCHED* Gene in Several Types of Sporadic Extracutaneous Tumors¹

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Abstract

Patients with basal cell nevus syndrome have a high incidence of multiple basal cell carcinomas, medulloblastomas, and meningiomas. Because somatic *PATCHED* (*PTCH*) mutations have been found in sporadic basal cell carcinomas, we have screened for *PTCH* mutations in several types of sporadic extracutaneous tumors. We found that 2 of 14 sporadic medulloblastomas bear somatic nonsense mutations in one copy of the gene and also deletion of the other copy. In addition, we identified missense mutations in *PTCH* in two of seven breast carcinomas, one of nine meningiomas, and one colon cancer cell line. No *PTCH* gene mutations were detected in 10 primary colon carcinomas and eighteen bladder carcinomas.

Introduction

BCNS³ (Online Mendelian Inheritance in Man #109400) is a rare autosomal dominant disease with diverse phenotypic abnormalities, both tumorous (BCCs, medulloblastomas, and meningiomas) and developmental (misshapen ribs, spina bifida occulta, and skull abnormalities; Ref. 1). The *BCNS* gene was mapped to chromosome 9q22.3 by linkage analysis of BCNS families and by LOH analysis in sporadic BCCs (2). LOH in sporadic medulloblastomas has been reported in the same chromosome region (3). Recently, the human homologue of the *Drosophila patched* (*PTCH*) gene has been mapped to the BCNS region (4-7), and mutations in this gene have been found in the blood DNA of BCNS patients and in the DNA of sporadic BCCs (4-6, 8). *PTCH* appears to function as a tumor suppressor gene; inactivation abrogates its normal inhibition of the hedgehog signaling pathway. Because of the wide variety of tumors in patients with the BCNS and wide tissue distribution of *PTCH* gene expression, we have begun screening for *PTCH* gene mutations in several types of human cancers, especially those present in increased numbers in BCNS patients (medulloblastomas), those in tissues derived embryologically from epidermis (breast carcinomas) and those with chromosome 9q LOH (bladder carcinomas; see Refs. 9 and 10).

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³The abbreviations used are: BCNS, basal cell nevus syndrome; BCC, basal cell carcinoma; LOH, loss of heterozygosity.

Materials and Methods

Clinical Materials. Diagnoses of all tumors were confirmed histologically. Cell lines were obtained from the American Type Culture Collection. DNA was extracted from tumors or matched normal tissue (peripheral blood leukocytes or skin) as described (11,12).

PCR and Heteroduplex Analysis. PCR amplification and heteroduplex/SSCP analysis were performed as described (5,13). Primers used and intron/exon boundary sequences of the *PTCH* gene were derived as reported previously (5) and are shown in Table 1. Primers for exon 1 and 2 were from Hahn *et al.* (4).

Sequence Analysis. Exon segments exhibiting aberrant bands were reamplified and were sequenced directly using the Sequenase sequencing kit according to the protocol recommended by the manufacturer (United States Biochemical Corp.). A second sequencing was performed using independently amplified PCR products to confirm the sequence change. The amplified PCR products from each tumor were also cloned into the plasmid vector pCR 2.1 (Invitrogen); followed by sequence analysis of at least four independent clones. The sequence alteration was confirmed from at least two independent clones. Simplified amplification of specific allele analysis was performed according to Lei and Hall (14).

Allelic Loss Analysis. Microsatellites used for allelic loss analysis were *D9S109*, *D9S119*, *D9S127*, *D9S196*, and *D9S287* described in the Cooperative Human Linkage Center human screening set (Research Genetics). A part of the *PTCH* intron 1 sequence was tested for polymorphism in a control population and found to be polymorphic in 80% of the samples tested. This microsatellite was used for analysis of *PTCH* gene allelic loss in bladder carcinomas. The primer sequences are as follows: forward primer, 5'-CTGAGCAGATTTC-CCAGGTG-3'; and reverse primer, 5'-CCTCAGACAGACGTTTCCTC-3'. The PCR cycling for this newly isolated marker was 4 min at 95°C, followed by 30 cycles of 40 s at 95°C, 2 min at 60°C, and 1 min at 72°C. PCR products were separated on 6% polyacrylamide gels and exposed to film.

Results and Discussion

Intronic boundaries were determined for 22 exons of *PTCH* by sequencing vectorette PCR products derived from BAC 192J22 (Ref. 5; Table 1). Our findings are in agreement with those of Hahn *et al.* (4), except that we find exon 12 is composed of two separate exons of 126 and 119 nucleotides. This indicates that *PTCH* is composed of 23 coding exons instead of 22. In addition, we find that exons 3, 4, 10, 11, 17, 21, and 23 differ slightly in size than reported previously (4). Of 63 tumors studied, 14 were sporadic medulloblastomas and 9 were sporadic meningiomas. These 23 tumors were examined for allelic deletions by genotyping of tumor and blood DNA with microsatellite markers that flank the *PTCH* gene: *D9S119*, *D9S196*, *D9S287*, *D9S127*, and *D9S109*. Four of 14 medulloblastomas had LOH. Two of the medulloblastomas, both of which had LOH, had mutations (med34 and med36; see Ref. 11), which are predicted to result in truncated proteins (Fig. 1A and Table 2). DNA samples from the blood of these patients lack these mutations, indicating that they both are somatic

Table 1 Primers and boundary sequences of *PTCH*

Exon	5' Boundary ^a	Nucleotide position ^b	Exon size	3' boundary ^a	Reading frame ^c	Primers
1	ND ^d	ND	ND	AAGlgnat	ND	
2	ND	202	193	AAGltaaga	3	
3	tgacag T	395	190	CAGltaagg	1	3F GAGTTTGACGTGATGTTGCTATTTC 3R ACCGCTTACCTGCTGCTC
4	tattag G	585	70	CAGlgtat	2	4F TGCACATAATTTCTTATTACAGTGA 4R TAAGGCACACTACTGGGGTG
5	tgacag A	655	92	CCTlgttaagt	3	5F GAACACCCAGTAGTGTGCC 5R TGAGTCTAGAGAAGTACAGACATC
6	ttgcag A	747	199	AAAlgtgagt	2	6F GGCTCTTTTCATGGTCTCGTC 6R TGTTTGTCTCCACCCCTTC
7	tttag C	946	122	CAGlgttaage	3	7F GCACGTGGATTTTAAACAGGCATG 7R AGGGGATAGATTGTCTCTCGG
8	ctgcag C	1068	148	GAGlgttaac	2	8F TGGGAATACTGATGATGTGCC 8R CATAAACAGCGAGTCTGCAC
9	ccacag G	1216	132	ATGlgttaacg	3	9F CATTTGGGCAATTCGCATTC 9R ACCAAACCAACTCCAGCCC
10	ttgcag C	1348	156	CAGlgttacta	3	10F TGCCCCCATTTGTTCGTGTTG 10R GGACAGCAGATAAATGGCTCC
11	ctgtag G	1504	99	GAGlgttaag	3	11F GCATCTCGCATGTCTAATGCCAC 11R AAGCTGTGATGTCCCAAG
12	tcacag G	1603	126	CAGlgtgagc	3	12F GACGATGTCCAGTGCAGCTC 12R CGTTTCAGGATCACCACAGCC
13	tcacag G	1729	119	AAGlgtacat	3	13F AGTCTCTGATTGGCGGAG 13R CCATTTCTGCACCCATCAAAAG
14	tttcag C	1848	403	AAGlgttaac	2	14F AAAATGGCAGAAATGAAGCACC 14R CTGATGAACCTCCAAAGTTCTG
15	ttccag G	2251	310	AGGlgttaaga	3	15F GGAAGAGTCACTGTGTCTCC 15R CGCCAAAGACCGAAAGGAC
16	ttctag G	2561	143	CAGlgttacc	1	16F AGGGTCTCTTGGCTGCGAG 16R GCTGTCAAGCAGCCTCCAC
17	ttgtag T	2704	184	GAAlgttaagt	3	17F GCTCTCAAGGCAGAAAGTGTG 17R GGAAGGCACCTCTGTAAAGTTC
18	gtccag T	2888	281	ATTlgtgagt	1	18F GCTCTTAACCTGTGCCCTTC 18R GAATTTGACTTCCACAAAGCCC
19	ctccag G	3169	138	TTGlgtatgg	3	19F CCCCCACTGACCACTGTGTG 19R GAGCCAGAGGAAATGGGTTG
20	gcacag G	3307	143	CAGlgttaage	3	20F AGCATTTACCAAGTGAAGTCC 20R TTGCACACGCTGCTTAC
21	tcacag G	3450	100	GAGlgticagt	2	21F TGTTCCCGTTTCTCTCTTG 21R GCACAGGAAACACAGCATTC
22	aatag G	3550	255	ACTlgttaagt	3	22F GCAGGTAAATGGACAAGAACAC 22R ACTACCACGGTGGGAAGACC
23	ctgcag G	3805	541	GAGlgtgagt	3	23F CCCTTCTAACCCAGCCTCAC 23R GACACATCAGCCTTGCTC
24	ND	4346	ND	ND		

^a Consensus sequences for the 5' and 3' exonic boundaries are (C₂)₁₁ncag|G and AGlgt₁₈agt, respectively (20). Upper case denotes exonic sequence.

^b Exon positions are in reference to the coding sequence of *PTCH* (3) with the beginning ATG as nucleotide 1.

^c 5' exon boundary begins after the first, second, or third base of the codon of the translational reading frame.

^d ND, not determined.

mutations. med34 also has allelic loss on 17p (11). We were unable to detect *PTCH* gene mutations by heteroduplex analysis in the other two medulloblastomas bearing LOH on 9q. The pathological features of these two tumors differed in that med34 belongs to the desmoplastic subtype, whereas med36 is of the classic type, indicating that *PTCH* mutations in medulloblastomas are not restricted to a specific subtype. One report (3) has shown that five medulloblastomas (two BCNS-associated cases and three sporadic cases) bearing LOH on chromosome 9q22.3-q31 are all of the desmoplastic subtype, suggesting LOH on 9q22.3 is histological subtype specific. We feel that the conclusion derived from only five positive tumors is not a strong one because we and others (15) have found nondesmoplastic subtypes of medulloblastomas bearing LOH on chromosome 9q22.3. Independently, another group has reported their finding of *PTCH* mutations in sporadic medulloblastomas (15).

A change of T to C at nucleotide 2990 (in exon 18) was identified in DNA from one of nine sporadic meningiomas, causing a predicted change of codon 997 from Ile to Thr (Table 2). The meningioma bearing this mutation also has allelic loss on 9q22.3. Blood cell DNA is heterozygous for this mutation, but DNA from the tumor contains only the mutant sequence. Of 100 normal chromosomes examined, none has this sequence change, suggesting that this mutation is not likely a common polymorphism. This patient is 84 years old and has

had no phenotypic abnormalities suggestive of the BCNS, suggesting that this sequence alteration may not have caused complete inactivation of the *PTCH* gene. None of the other eight meningiomas had detectable LOH at chromosome 9q.

We also screened for *PTCH* gene mutations in DNA from 7 breast carcinomas (four primary tumors and three cell lines), 11 colon tumors (10 primary tumors and 1 cell line), 18 bladder tumors (14 primary tumors and 4 cell lines), and 2 ovarian cancer cell lines. These tumors are not known to occur in higher than expected frequency in BCNS patients. We identified sequence abnormalities in two breast carcinomas and in the one colon cancer cell line (Table 2). The mutation found in breast carcinoma Br349 is not present in the patient's normal skin DNA, indicating that the sequence change is a somatic mutation (Fig. 1B). Direct sequencing of the PCR product indicated that only the mutant allele is present in the tumor. This mutation changes codon 955 from Tyr to His, and this Tyr is conserved in human, murine, chicken, and fly *PTCH* homologues (16). The mutation in breast carcinoma Br321 is predicted to change codon 995 from Glu to Gly, and the tumor with this mutation retains the wild-type allele. We have sequenced exon 18 in DNA from the blood of 50 normal persons and found no changes from the published sequence, suggesting that the sequence change found in Br321 is not a common polymorphism. Furthermore, examination of the DNA

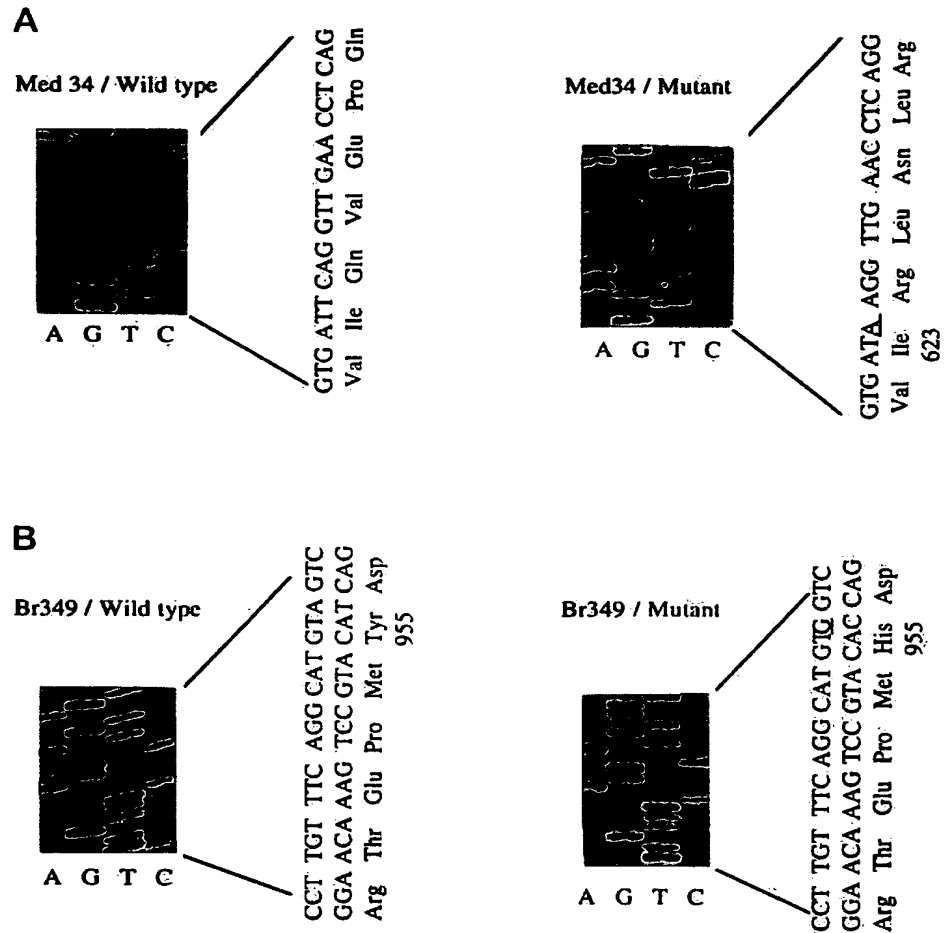


Fig. 1. Sequence analysis of *PTCH* mutations. A, the sense strand sequence of tumor med34 has a TC to A change in exon 14, which creates a stop codon at codon 623. B, the antisense strand sequence of tumor Br349 exhibits a mutation of G to A in exon 17, which causes a change of Tyr to His in the coding sequence.

from the cultured skin fibroblasts of the patient did not reveal the same mutation, indicating that this is a somatic mutation. Although mutations of the other allele were not detected by heteroduplex/SSCP analysis in tumor Br321, we believe our mutation screening does not identify all mutations; therefore, we postulate that this sequence change is related to the patient's pathological condition. Neither Br321 nor Br349 tumors have recurred following their surgical removal, although four of eight lymph nodes were positive for Br321 at the time of mastectomy. Because DNA is not available from normal cells of the patient from which colon cell line 320 was established, we used simplified amplification of specific allele analysis (14) to examine 50 normal blood DNA samples for the presence of the sequence

alteration and found none but the DNA from this cell line to have the mutant allele, suggesting that this mutation also is unlikely to be a common sequence polymorphism. For bladder carcinomas, a newly isolated microsatellite that was derived from intron 1 of the *PTCH* gene was used to examine LOH in the tumor. Three primary bladder carcinomas showed LOH at this intragenic locus. With no *PTCH* mutations detected in these tumors, we suspect that the LOH in these three bladder carcinomas may reflect the high incidence of whole chromosome 9 loss in bladder cancers (10). A similar observation has been reported previously (17).

We also detected a sequence change in intron 10 in two colon carcinomas, 15-1 and 8-1, an alteration that was reported previously

Table 2. *PATCHED* gene alterations^a

Tumor	Pathology	Nucleotide	Codon	Exon	Consequence	LOH	Mutation type
Med34	Medulloblastoma (desmoplastic)	TC1869A	623	14	Frameshift	Yes	Somatic
Med36	Medulloblastoma (classic)	G2503T	835	15	Glu to STOP	Yes	Somatic
Men1	Meningioma	T2990C	997	18	Ile to Thr	Yes	Germ-line
Br349	Breast carcinoma	T2863C	955	17	Tyr to His	Yes ^b	Somatic
Br321	Breast carcinoma	A2975G	995	18	Glu to Gly	No	Somatic
Co320	Colon tumor cell line	A2000C	667	14	Glu to Ala	No ^b	Unknown
Co8-1	Colon carcinoma	T to C ^c	Intron 10		Polymorphism	No	Germ-line
Co15-1	Colon carcinoma	T to C ^c	Intron 10		Polymorphism	No	Germ-line

^a All patients with *PATCHED* alterations have no clinical symptoms indicative of basal cell nevus syndrome.

^b Determined by direct sequencing of the tumor DNA, which shows only the mutant (Yes) or both mutant and wild-type alleles (No).

^c The position of this polymorphism is 8 bp from the intron 10-exon 11 boundary.

as a splicing mutation (18). Because we found the same sequence change in about 20% of normal control samples, we suggest that this more likely is a nonpathogenic polymorphism. The *PTCH* protein is predicted to contain 12 transmembrane domains, two large extracellular loops, and one intracellular loop (16). Of the six mutations we identified, four are missense mutations. Three mutations lead to amino acid substitutions in the second extracellular loop, and one mutation results in an amino acid change in the intracellular domain.

Our data indicate that somatic inactivation of the *PTCH* gene does occur in some sporadic medulloblastomas. In addition, because missense mutations of the *PTCH* gene were detected in breast carcinomas, we suspect that defects of the *PTCH* function also may be involved in some breast carcinomas, although biochemical evidence is necessary to show how these missense mutations might impair *PTCH* function. Of 11 colon cancers and 18 bladder carcinomas examined, we found only one mutation in 1 colon cell line, suggesting that *PTCH* gene mutations are relatively uncommon in colon and bladder cancers, although the incidence of chromosome 9 loss in bladder cancers is high (9).

Published reports of SSCP analysis of tumor DNA identified mutations in the *PTCH* gene in only 30% of sporadic BCCs, although chromosome 9q22.3 LOH was reported in more than 50% of these tumors (6). It has been reported that heteroduplex/SSCP analysis of gene mutations is more sensitive than SSCP analysis (13). In our studies, we were able to identify a point mutation in the 310-bp PCR product from exon 15 using heteroduplex analysis, whereas SSCP analysis failed to reveal this sequence change (Table 2). Therefore, we suspect that there may be more mutations in BCCs than we have found thus far. Analysis of the *PTCH* gene in BCNS patients and in sporadic BCCs has identified mutations scattered widely across the gene, and the majority of mutations were predicted to result in truncated proteins (4–6, 8, 18, 19). In our screening, we found two breast carcinomas bearing missense mutations of the *PTCH* gene. In one of these two tumors, B349, direct sequencing indicated a deletion of the other copy of the *PTCH* gene. Any comparison of mutations in skin cancers versus extracutaneous tumors must consider the wholly different causes of these mutations; UV light is unique to the skin.

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Trichoepitheliomas Contain Somatic Mutations in the Overexpressed *PTCH* Gene: Support for a Gatekeeper Mechanism in Skin Tumorigenesis¹

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Abstract

The nevoid basal cell carcinoma (Gorlin) syndrome (NBCCS) is an autosomal dominant disorder characterized by multiple developmental defects and cancer susceptibility. NBCCS is caused by mutations in the human homologue (*PTCH*) of the *Drosophila patched* gene, a developmental regulator implicated in signaling of *hedgehog* and *smoothened*. The *PTCH* gene was found to contain somatic mutations also in sporadic basal cell carcinomas and medulloblastomas, tumors seen in NBCCS, consistent with *PTCH* acting as a tumor suppressor. Because basal cell carcinomas have been observed to develop in association with benign trichoepitheliomas (TEs) in the same lesions, patients, and families and may share the same cell of origin, we have analyzed *PTCH* for mutations and expression in TEs. We report frameshift and in-frame somatic deletions in this gene and a consistent overexpression of *PTCH* mRNA in TEs. These findings provide the first evidence of a gene mutation in TEs and identify a common pathogenic pathway for histopathologically similar but prognostically distinct skin tumors. Moreover, these results support the presence of a gatekeeper mechanism in multistep skin tumorigenesis exerted by the altered *PTCH* signaling pathway.

Introduction

The NBCCS⁴ (McKusick number MIM 109400; for a review see Ref. 1) is an autosomal dominant disorder characterized by multiple developmental abnormalities and susceptibility to a variety of tumors, in particular to BCCs. BCC of the skin is the most common cancer in humans, and its annual incidence has increased considerably in the last 2 decades (2). BCCs seen in NBCCS develop earlier in life and are often seen in large numbers at multiple sites, consistent with the NBCCS gene acting as a tumor suppressor. The NBCCS locus was mapped to the long arm of chromosome 9 by genetic linkage analysis, supported by frequent loss of constitutional heterozygosity at 9q (3-5), and the defective gene was found by positional cloning (6-8). The gene was identified as *PTCH*, a human homologue of *ptc*, an important developmental regulator, initially found as a segment polarity gene (9). *ptc* was implicated in the transcriptional repression of genes encoding members of the transforming growth factor- β and Wnt proteins and in the signaling pathway of *Drosophila hedgehog* and *smoothened* (10-13). The human gene (*PTCH*) is expressed in target tissues of *shh* (6) and encodes a membrane receptor containing

12 transmembrane domains (7, 8) and two large extracellular loops that are essential for binding *shh* (10, 11).

Germ-line mutations of *PTCH* were identified in a significant proportion of families with NBCCS (7, 8, 14-16), but they do not appear to show any obvious phenotypic correlates (14). A number of observed mutations, which were accompanied by no copy of the wild-type sequence in BCCs of NBCCS patients, would be predicted to result in a loss of function of the gene product (7, 8, 14-16). Mutations have also been found in sporadic BCCs (7, 8, 15-17), in which *PTCH* was found invariably overexpressed as analyzed by *in situ* hybridization (17, 18). Recent studies indicated that this gene is also mutated in medulloblastomas (19, 20), which are primitive neuroectodermal tumors and are considered to constitute a diagnostic feature of NBCCS (1). Recently, Oro *et al.* (21) reported a putative activating missense mutation (H133T) in human *shh* in several tumors, suggesting that *PTCH* may be a part of an important cancer pathway. The inactivation of the *PTCH* gene product was proposed to be virtually necessary for passing the genetic threshold of the neoplastic process in the skin (22).

TEs are benign skin tumors with follicular differentiation, most commonly seen on the face and sometimes urging cosmetic interventions. They appear as skin-colored nodules or papules with few telangiectasias. If they present as multiple lesions, they are usually smaller and are commonly found on the nasolabial folds and upper lips (23, 24). These tumors are occasionally familial, usually transmitted from one generation to another with no clear sex preference, compatible with autosomal dominant inheritance. Interestingly, BCCs have been reported in such families and both tumor types have been suggested to have a common origin in hair follicle keratinocytes (23, 24). Although there is a defined set of histopathological and clinical diagnostic criteria, TEs can, in some cases, be mistaken for BCCs and *vice versa* (23, 24). To test *PTCH* as a candidate gene involved in the development of TEs, we have analyzed this gene for mutations using SSCP analysis of genomic DNA extracted from tumor cells dissected from TEs and for expression by *in situ* hybridization.

Materials and Methods

Tumor Samples. A total of 21 different TEs derived from 17 unrelated patients were analyzed for *PTCH* mRNA expression and CD34 staining. Nine tumor samples from unrelated Swedish patients with TEs were included in the mutational analysis. The samples were obtained from the Karolinska and Danderyd Hospitals (Stockholm, Sweden). The mean age of patients at diagnosis was 55 years and ranged from 25 to 82 years. With the exception of two patients with multiple TEs (TR4 and TR8 in Table 1), none of the patients had a known positive family history of TEs. Patient TR4 had an affected brother with multiple TEs, and patient TR8 had a sister and a daughter with multiple TEs. The patients and tumors are described in Table 1. All tumors were histologically reviewed and classified according to established criteria (23). All tumors were reevaluated independently, and only clinically and histopathologically typical TEs were included. All TEs were small, skin-colored, nonulcerated papules. They were epithelial-mesenchymal lesions with frond-like epithelial growth patterns, surrounded by fibroblastic stromas forming abortive or

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⁴ The abbreviations used are: NBCCS, nevoid basal cell carcinoma (Gorlin) syndrome; BCC, basal cell carcinoma; *ptc*, *Drosophila melanogaster patched*; *PTCH*, human homologue of *ptc*; SHH, sonic hedgehog; TE, trichoepithelioma; SSCP, single-strand conformation polymorphism; CC, cutaneous cylindroma.

Table 1 Clinical characterization of TEs and *in situ* PTCH mRNA expression

Sample	Sex	Tumor location	Ages at diagnosis/sampling (yr)	Signal intensity by ISH ^a
TR1 ^b	M	Periorbital	59/59	++
TR4	M	Forehead ^c	30/79	+
TR5	F	Upper eyelid	64/64	++
TR6	M	Forehead	82/82	++
TR7	F	Periorbital	50/50	+
TR8	F	Nasolabial ^c	25/49	+
TR10	F	Nasolabial	49/49	++
TR11 ^b	M	Forehead	41/41	++
TR12	F	Nose	44/44	++

^a Signal intensity was estimated using a semiquantitative scale from 0 to ++.^b Tumors with *PTCH* mutations.^c Denotes samples from patients with multiple and familial TEs; the remaining tumors were solitary and sporadic.Table 2 Summary of *PTCH* mutations in TE DNA samples

Sample	Primers	<i>PTCH</i> exon	Nucleotide change ^a	Protein change ^a	Presence of the wild-type allele	Mutation in the germ line
TR11	PTC20	20	3509del26, ^b 3539del11	L1170W, delL1171-G1179, P1180T	45%	Absent
TR1	PTC7	8	1108delAAGC	Frameshift	<1%	Absent

^a The numbering of nucleotides and codons is as in Ref. 8 (GenBank accession no. U59464).^b The deleted sequence was 5'-TGC TTC CCG TGC TTT TGT CTT TCT TT-3', followed by 3539delC.

developed hair bulb structures. All TEs contained small keratinocysts. Cellular material was punched out from macro- and microscopically well-defined nodules in the paraffin blocks to minimize contamination with normal cells.

DNA Extraction. DNA was extracted from paraffin-embedded material using methods described previously (25, 26). Constitutional DNA was isolated either from patients' blood samples or normal fixed tissue using the same methods.

PCR-SSCP Analysis and Sequencing. The sequence of oligonucleotide primers used for PCR-SSCP were as described previously (7, 16). The sequences of the forward and reverse primers flanking the *shh* H133T mutation (21) were 5'-GAG GGC TGG GAC GAA GAT GG-3' and 5'-CTG CGG TCG CGG TCA GAC G-3', respectively. PCRs contained 100 ng of DNA, 0.25 μ M each primer, 100 μ M each dNTP, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.2 μ Ci of [α -³²P]dCTP (3000 Ci/mmol), and 0.5 units of Taq polymerase (Perkin-Elmer) in a volume of 20 μ L. Amplifications were performed for 30–35 cycles, with denaturation at 94°C for 30 s, annealing at 52–58°C for 45 s, and extension at 72°C for 30 s in a Perkin-Elmer thermocycler (GeneAmp System 9600). The samples were mixed with 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 50 mM NaOH, denatured at 95°C for 10 min, and loaded onto 0.4 mm $\times$ 30 cm $\times$ 45 cm 5% polyacrylamide-5% glycerol gels (cross-linking, 2.5%). The electrophoresis was conducted at 2–5 W at room temperature overnight. The gels were dried and autoradiographed for 1–2 days at –70°C. *PTCH* segments identified as containing mutations were reamplified in parallel from the original DNA sample, positive and negative controls, and fragments exhibiting bandshifts cut from the SSCP gels (26). DNA was sequenced in both directions using cycle sequencing with AmpliTaqFS (Perkin-Elmer) on the Applied Biosystems 373A DNA sequencer.

In Situ Hybridization. *In situ* hybridization and preparation of *PTCH* RNA probes were carried out as described previously (14, 18). Briefly, paraffin-embedded tumor sections from 21 TEs were treated with proteinase K and washed in 0.1 M triethanolamine buffer containing 0.25% acetic anhydride. Sections were hybridized with 2.5 $\times$ 10⁶ cpm of labeled antisense or sense probe at 55°C. Autoradiography was performed for 2 weeks.

Immunohistochemistry. A mouse monoclonal antibody directed against CD34 (QBEND/10; Unipath, Basingstoke, United Kingdom), diluted 1:50, was used. Sections from each tumor sample were incubated with antibody for 16 h at room temperature and processed using a standard avidin-biotin immunoperoxidase/diaminobenzidine detection system. Control serial sections were used with no primary antibody.

Results

Identification of *PTCH* Mutations in TEs. The *PTCH* coding region was amplified from tumor DNA samples using oligonucleotide primer pairs flanking each exon. The PCR products were analyzed on

SSCP gels, and those with altered mobility were sequenced. Mutations were found in two patients (Table 2 and Fig. 1). Although one sample (TR1 in Table 1 and Fig. 1A) had a frameshift deletion with no detectable wild-type *PTCH* allele, the other sample with a mutation was found to contain significant amounts of the normal copy of *PTCH* (patient TR11 in Table 1 and Fig. 1B). The latter mutation was a larger deletion in exon 20 and would be predicted to lead to a removal of the COOH-terminal part of the 12th transmembrane domain. This domain was previously shown to be deleted and mutated in tumor DNA extracted from BCCs and medulloblastomas (16, 20) and is likely to be essential for downstream signaling by *PTCH*. The ratio of the signal intensity from the wild-type and mutated allele was estimated at about 45% (Fig. 1B).

Somatic Origin of *PTCH* Mutations. To determine whether these mutations are present in the germ line, normal DNA samples from patients TR1 and TR11 were analyzed in parallel with tumor DNA and controls. The results revealed no copy of the mutated allele in the germ line (data not shown), indicating a somatic origin of the mutations. Thus, the significant amounts of normal copy observed in sample TR11 was due to the presence of cells not containing the alteration, presumably normal stromal cells. No mutations were found in tumor or normal DNA from patients demonstrating multiple TEs (samples TR4 and TR8 in Table 1).

Screening for the H133T Mutation in *SHH*. In *Drosophila*, an excess of *hedgehog* function can have an effect similar to loss of *ptc* function, and this relationship appears to be conserved in vertebrates. Transgenic mice overexpressing *shh* in the skin develop many features of NBCCS, including BCC-like tumors (21). One candidate activating mutation (H133T) in the human *shh* was recently found in 1 of 43 BCCs, 1 of 14 medulloblastomas, and in 1 of 6 breast cancers (21). We have examined TE tumor DNA for this particular change; however, the mutation was not found in any samples, nor was it present in any tumor DNA extracted from 32 unrelated patients with primitive neuroectodermal tumors or in five medulloblastoma cell lines.⁵

Expression of *PTCH* mRNA. The results of *in situ* hybridization experiments are shown in Table 1 and Fig. 2. All TEs included in the mutation screening showed CD34 positivity (Fig. 2F), confirming

⁵ I. Vofechovský, O. Tingby, M. Nistér, V. P. Collins, and R. Toftgård, unpublished data.

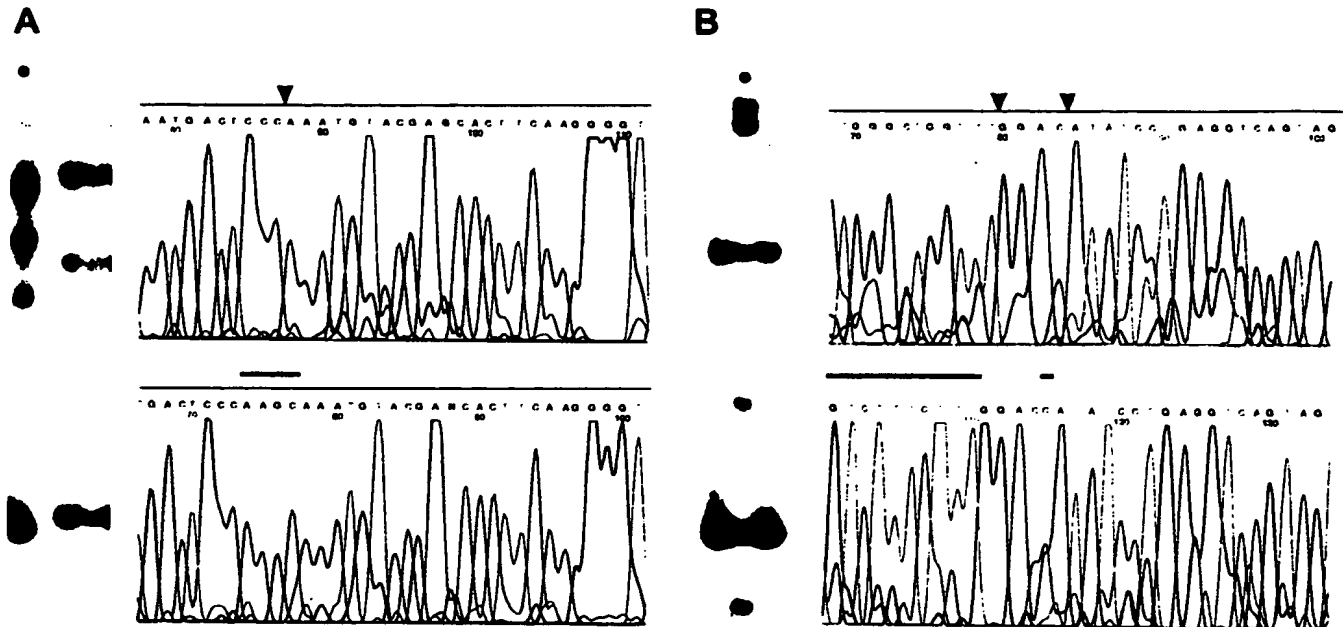
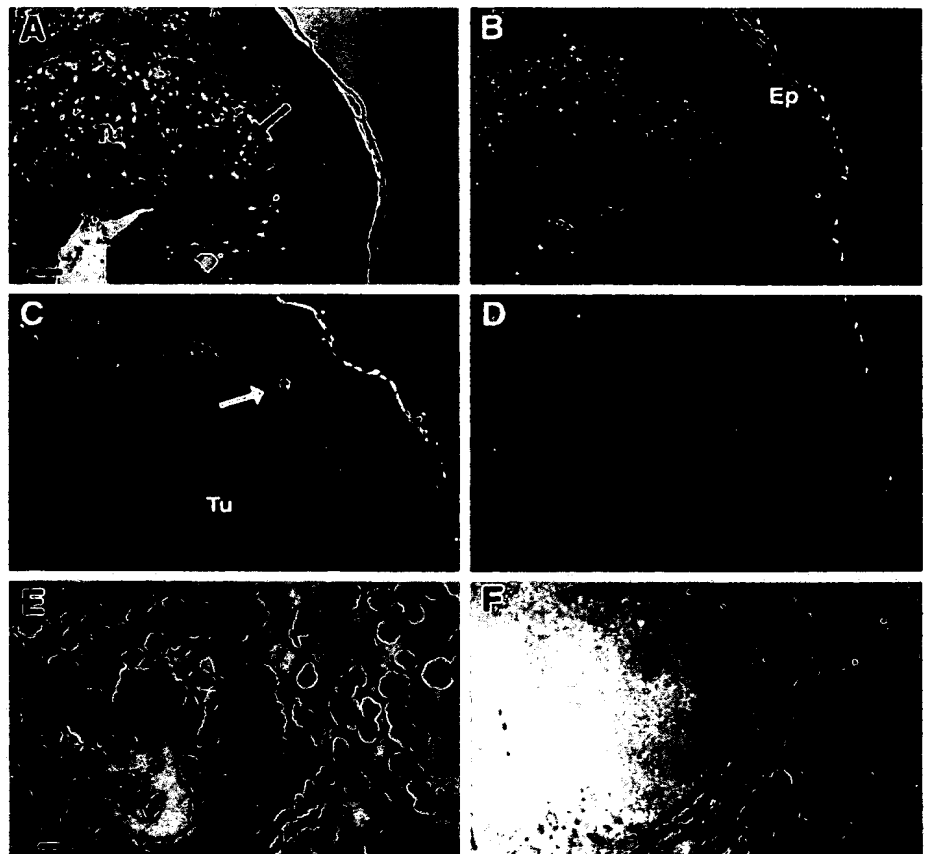


Fig. 1. SSCP alterations in tumor DNA samples of two patients with TEs and a corresponding nucleotide sequence. A, patient TR1, exon 8; B, patient TR11, exon 20. *, abnormal SSCP patterns. Arrowheads, deletion breakpoints in patients (top); bars, missing nucleotides in controls (bottom). Second arrowhead in exon 20 of patient TR11 (B), a cytosine deletion, which restores the reading frame.

Fig. 2. Expression of *PTCH* mRNA and CD34 staining in a solitary TE. A, bright-field photomicrograph of TE counterstained with H&E. Tu, tumor cells. Arrow, area shown in E under high magnification. B, dark-field photomicrograph of the same section. The tumor sections were hybridized with ³⁵S-labeled antisense *PTCH* probe. Abundant autoradiographic signal is seen in tumor cells, whereas no expression is observed in normal epidermis (Ep). C, dark-field photomicrograph of an adjacent area of the same tumor. In contrast to the strong signal for *PTCH* mRNA in tumor nests (Tu), no signal is detected in normal follicular epithelium (arrow). D, sections of the same tumor hybridized with the sense probe showed no signal. E, tumor nests under high power demonstrate specific signal manifested as black grains in tumor cells. F, positive immunoreactivity for anti-CD34 antibody in stromal cells adjacent to tumor cells. Bars, 150 μ m (A-D and F) and 15 μ m (E).



previous findings (27) and, together with clinical and histopathological criteria, indicating that the tumors analyzed were TEs and not BCCs. The analysis of 21 TEs revealed a strong *PTCH* signal confined to tumor cells in 19 TE sections, whereas two TEs of the desmoplastic variant lacked overexpression. No detectable signal could be seen in normal epidermis or normal follicular epithelium (Fig. 2, B and C). All samples included in mutation screening showed an overexpression, and the mutated tumor samples showed a relatively strong signal intensity (Tables 1 and 2). The average signal intensity from TE tumor cells was comparable to that previously observed in BCCs (17, 18). These results present the first evidence of a consistently overexpressed gene in benign tumors of the skin and suggest that *PTCH* overexpression reflects a failure in the negative feedback mechanism due to mutations in *PTCH* or possibly in other genes in the signaling cascade.

Discussion

Here, we provide the first demonstration of the involvement of the important tumor suppressor gene *PTCH* in benign skin tumorigenesis. Our finding of two of nine mutated TEs suggests that altered *PTCH* or, more generally, alterations in the signaling pathway leading to overexpression of *PTCH* may be required for the initiation of both BCCs and TEs. One of the tumors (TR1 in Table 2) exhibited the frameshift mutation and virtual absence of normal copy of *PTCH*, indicating somatic inactivation of the gene in this tumor, which is consistent with *PTCH* acting as tumor suppressor. Our results are compatible with previously formulated hypothesis postulating the existence of a gatekeeper mechanism in skin tumor development (22), originally suggested for the inactivated *APC* gene in the initiation of colorectal tumors (28). As in dominantly inherited familial adenomatous polyposis, which typically leads to the development of hundreds to thousands of adenomas, NBCCS alleles confer extremely high risks of developing similar numbers of BCCs. The Knudson's "second hit" in a cell already containing a single NBCCS allele seems to be most commonly represented by a mitotic recombination event but can also be achieved by a somatic mutation, loss of large chromosomal regions (16, 20), or other mechanisms. This event may be rate limiting in the development of these and other epithelial tumors. By contrast, squamous cell carcinomas of the skin, lungs, and esophagus have not been found to contain *PTCH* mutations (29, 30), nor have they been found in tumors of mesenchymal origin (31). Recent data show *PTCH* mutations also in extracutaneous ectodermal tumors and in additional tumors seen in NBCCS (32).

Consistent with our previous observations in BCCs (17, 18), an increased expression of *PTCH* at the level of RNA seen in all but two TEs analyzed, including tumors with somatic mutations, suggests that deregulation of the SHH-*PTCH* signaling pathway may be an early and necessary event in TE development. Interestingly, two TEs that did not show *PTCH* mRNA overexpression were of the desmoplastic type. Because a susceptibility to multiple desmoplastic TEs has been described as a separate hereditary syndrome (McKusick number MIM 190345; Ref. 33), the development of desmoplastic TEs may result from accumulating distinct genetic events.

There has been a controversy as to the origin of BCCs and TEs. Multiple hereditary TEs were reported independently by Brooke (34) and Fordyce (35) as epithelioma adenoides cysticum. Previously reported cases of malignant transformation of TEs have later been considered BCCs in NBCCS (36). Association of BCCs and multiple hereditary TEs without other signs of NBCCS was described, but the occurrence of BCCs and TEs in the same patient has only been well documented in a few reports (37–39). However, Johnson and Bennett (38) suggested that the association of BCCs and TEs is not unusual,

and histopathological characteristics of the two tumor types support the notion that they are related. The absence of *PTCH* mutations and overexpression in squamous cell carcinomas (29, 30) and their presence in BCCs and TEs may argue in favor of their common histogenetic origin, perhaps supporting the follicular hair cells as a source of malignant transformation. In addition to common *PTCH* alterations, further genetic changes may be present in BCCs or TEs or both that would account for distinct biological potential of the two tumor types.

The development of hair follicle-derived tumors, including TEs and tricholemmomas, was reported in a nonclassical Li-Fraumeni syndrome patient with a constitutional missense mutation in exon 8 (R273C) of the *p53* gene (40). Positive nuclear immunostaining for *p53* was observed in tumor cells but not in surrounding normal cells, suggesting a possible presence of an additional somatic mutation in *p53*. However, to our knowledge, TEs have not been analyzed for mutations in this gene.

Using two African-American families, the tentative disease locus for multiple familial TE was recently mapped to 9p21, reporting a maximum LOD score of 3.31 at zero recombination fraction for D9S171, whereas the analysis of four markers at 9q22–q31 did not show a significant evidence for linkage (41). Although the number of genotyped family members was limited, these results, if confirmed, would support the existence of a TE susceptibility locus on chromosome 9p. Therefore, samples from well-documented multigenerational families should be collected, followed by genetic linkage analysis. In this context, it is worth noting that TEs have not been reported in NBCCS and that we did not find any *PTCH* mutations in the two patients with familial multiple TEs (Tables 1 and 2).

In addition to BCCs, TEs have been observed in the same patients together with multiple CCs, an autosomal dominant disorder known as the Brooke-Spiegler syndrome (42). A recent review of the literature on CC revealed that these tumors often appear concomitantly with TEs in sporadic patients (43). The same report documented a large family with a dominant transmission of these lesions, involving 20 family members with CCs only, 3 patients with TEs only, and 4 patients with the concomitant presence of both TEs and CCs (43). Furthermore, familial cases of CCs observed in the literature had lesions on the face, a typical location of TEs (43). CCs and TEs have even been reported together in a single congenital plaque lesion (42). Also, co-occurrence of multiple TEs and multiple eccrine spiradenomas have been observed in the same patients (44, 45). These observations strongly suggest a common genetic pathway in the development of TEs, spiradenomas, and CCs, and it will be interesting to examine DNA from these skin tumors for *PTCH* alterations. It is tempting to speculate that the genes altered in these tumor types will play a role in the SHH-*PTCH* signaling pathway.

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Application No: 10/682584
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For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit H

Maesawa C et al, *Genes Chromosomes Cancer* 1998;21:276-279.

BRIEF COMMUNICATION

Mutations in the Human Homologue of the *Drosophila* Patched Gene in Esophageal Squamous Cell Carcinoma

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The human homologue (*PTCH*) of the *Drosophila* segment polarity gene *patched* has recently been identified as a tumor-suppressor gene for nevoid basal cell carcinoma syndrome and for sporadic basal cell carcinomas of the skin. We analyzed 30 esophageal squamous cell carcinomas (ESCC) for intragenic mutations of the *PTCH* gene by polymerase chain reaction–single-strand conformation polymorphism analysis followed by DNA sequencing. We identified two somatic *PTCH* mutations (7%) in 30 ESCC. These were a nonsense mutation (CAG to TAG at codon 361) in exon 8 and a missense mutation (CAG to CTG, Gln to Leu at codon 816) in exon 14. These tumors exhibited loss of heterozygosity at the polymorphic site of the *PTCH* gene. These results indicate that inactivation of the *PTCH* gene via a two-hit mechanism occurs in a subset of ESCC. *Genes Chromosomes Cancer* 21:276–279, 1998. © 1998 Wiley-Liss, Inc.

The *patched* (*ptc*) gene is a member of the *Drosophila* segment polarity gene, which encodes a transmembrane protein that is produced in precise spatial patterns and is conserved in eukaryotic organisms (Nüsslein-Volhard and Wieschaus, 1980). Recently, the human homologue of *ptc* (termed the *PTCH* gene, formerly *PTC*) was identified on 9q22.3 (Hahn et al., 1996a,b; Johnson et al., 1996), in which the nevoid basal cell carcinoma syndrome (NBCCS) locus was mapped by linkage analysis (Gailani et al., 1992). NBCCS is an autosomal dominant disorder characterized by multiple developmental defects and susceptibility to a variety of tumors, in particular to basal cell carcinoma (BCC) (Gorlin, 1987). Germline mutations of the *PTCH* gene were found in patients with NBCCS, and somatic mutations were detected in sporadic BCC (Chidambaram et al., 1996; Gailani et al., 1996; Hahn et al., 1996a; Johnson et al., 1996; Undén et al., 1996). Therefore, the *PTCH* gene is a tumor-suppressor gene for BCC.

Esophageal squamous cell carcinoma (ESCC) and BCC share several genetic abnormalities; in particular, loss of heterozygosity (LOH) on 9q is the most common genetic alteration in both tumors (Aoki et al., 1994; Mori et al., 1994). Therefore, the *PTCH* gene may also play a role in esophageal carcinogenesis. We analyzed intragenic mutations and allelic deletion of the *PTCH* gene in sporadic ESCC by using polymerase chain reaction–single-

strand conformation polymorphism (PCR-SSCP) analysis.

Thirty primary tumors and matching normal tissues from patients with sporadic ESCC were obtained from the surgical service of the Iwate Medical University School of Medicine (Morioka, Japan). Genomic DNA was extracted directly from surgical materials by standard proteinase K digestion and phenol/chloroform extraction.

Twenty-five sets of primers for amplification of the *PTCH* gene were synthesized as previously described (Hahn et al., 1996a,b; Undén et al., 1996). Two hundred nanograms of genomic DNA were amplified as previously described (Hahn et al., 1996a,b; Undén et al., 1996). Five microliters of each PCR product were diluted 10-fold with gel-loading buffer (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol, 0.025 bromophenol blue) and heated at 95°C for 2 min. The samples were electrophoresed on a 6% neutral polyacrylamide gel, with or without 10% glycerol, at 5–40 W for 3–10 hr.

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TABLE 1. Summary of *PTCH* Mutations

Case	Exon	Nucleotide change ^a	Consequence ^b	LOH ^c
E-66	8	C 1069 T	Q 361 stop	+
E-68	8	C 1125 T	Y 379 Y	-
E-94	14	A 2435 T	Q 816 L	+

^aNucleotide number of Hahn et al. (1996).

^bAmino acid number of Johnson et al. (1996).

^cLOH (loss of heterozygosity) was determined by PCR-SSCP analysis at the polymorphic sites obtained by Gailani et al. (1996) or by the present authors.

PCR products eluted from the shifted bands detected by SSCP analysis were sequenced directly by using a terminator cycle sequencing kit (TaQ DyeDeoxy, Applied Biosystems) and an automated DNA sequencer (Model 373S, Applied Biosystems). The same primers were used in PCR amplification for SSCP analysis and cycle sequencing.

Somatic mutations were detected in 2 (7%) of the 30 primary ESCC by SSCP analysis (Table 1, Fig. 1). These mutations were a nonsense mutation (CAG to TAG at codon 361, Gln to stop) in exon 8 and a missense mutation (CAG to CTG, Gln to Leu at codon 816) in exon 14 (Fig. 1). A silent mutation was detected in exon 8 (TAC to TAT at codon 379, Tyr) (Fig. 1). We also identified a sequence polymorphism differing from those of Gailani et al. (1996). This was a base substitution (A/G) in the alternative first exon (exon 1A identified by Hahn et al., 1996b). Seventeen of the 30 ESCC were informative for LOH at the polymorphic sites identified by Gailani et al. (1996) or by the present authors. Eight (47%) of the 17 informative cases exhibited LOH, including the tumors with nonsense or missense mutation (Fig. 1).

Recent studies have demonstrated that the protein encoded by *ptc* is a receptor for sonic hedgehog (Shh) (Marigo et al., 1996; Stone et al., 1996), which is essential for patterning and growth during vertebrate development (Roelink et al., 1995). Stone et al. (1996) demonstrated that the PTC protein also binds to Smoothened (Smo), which is an essential component in the hedgehog (Hh) pathways; mutation of Smo results in the same phenotype as does an Hh mutant (Roelink et al., 1995). However, Smo does not bind directly to the Hh (Stone et al., 1996). Thus, the investigators hypothesized that PTC is a ligand-binding component, whereas Smo is a signaling component in the (Shh-PTC-Smo) multisubunit complex (Stone et al., 1996). In this system, PTC acts as a ligand-regulated suppressor of the

(Smo signaling) unit (Stone et al., 1996), and, if PTC is disrupted, Smo is constitutively activated (Stone et al., 1996). Therefore, mutations leading to a truncated or unstable PTC protein could provide mitogenic or differentiation signals to basal cells in the skin throughout life (Stone et al., 1996). Stone et al. (1996) also suggested that NBCCS and BCC may result from constitutive activation of Smo, which is oncogenic when not controlled by PTC.

The incidence of *PTCH* gene mutations is generally low in carcinomas other than BCC. Simoneau et al. (1996) screened 69 transitional cell carcinomas of the urinary bladder for mutations of the *PTCH* gene and failed to detect mutations. This result was supported by Xie et al. (1997). No mutation was detected in 10 sporadic colorectal carcinomas (Xie et al., 1997). In medulloblastomas, the incidences were reported to be 13% (3 of 24, by Raffel et al., 1997) and 14% (2 of 14, by Xie et al., 1997). Suzuki et al. (1997) failed to detect *PTCH* mutations in 20 ESCC; however, in the present study, we demonstrated two ESCC (2/30, 7%) in which the *PTCH* gene is inactivated via the classic two-hit mechanism. Although the incidence of intragenic mutations in ESCC was relatively low, inactivation of *PTCH* function may be important in a subset of ESCC.

Mori et al. (1994) detected frequent LOH on 9q (approximately 50%) in ESCC and in precursor lesions. They hypothesized that an important tumor-suppressor gene responsible for ESCC may be located on 9q (Mori et al., 1994). Explanations for the low incidence of *PTCH* mutations are (1) another targeted gene resides on 9q and (2) other mechanisms act to inactivate the *PTCH* gene. The growth-arrest-specific gene *GAS1* has been mapped to 9q21.3-22 (Del Sal et al., 1994). Miura et al. (1995) detected a common deleted region in ESCC at 9q31-32, where the multiple self-healing epithelioma (*ESS1*) locus has been found (Pericak-Vance et al., 1995), but the responsible gene has not yet been identified. Abnormalities of these genes have yet to be evaluated in ESCC. In addition, homozygous deletion and de novo methylation of 5' CpG islands have been reported as another mechanism of inactivation of tumor-suppressor genes (Merlo et al., 1995). In fact, in ESCC, these alterations in the *CDKN2* gene on chromosome arm 9p are more prevalent than are intragenic mutations in ESCC (Maesawa et al., 1996). These genetic abnormalities in the *PTCH* gene require further investigation.

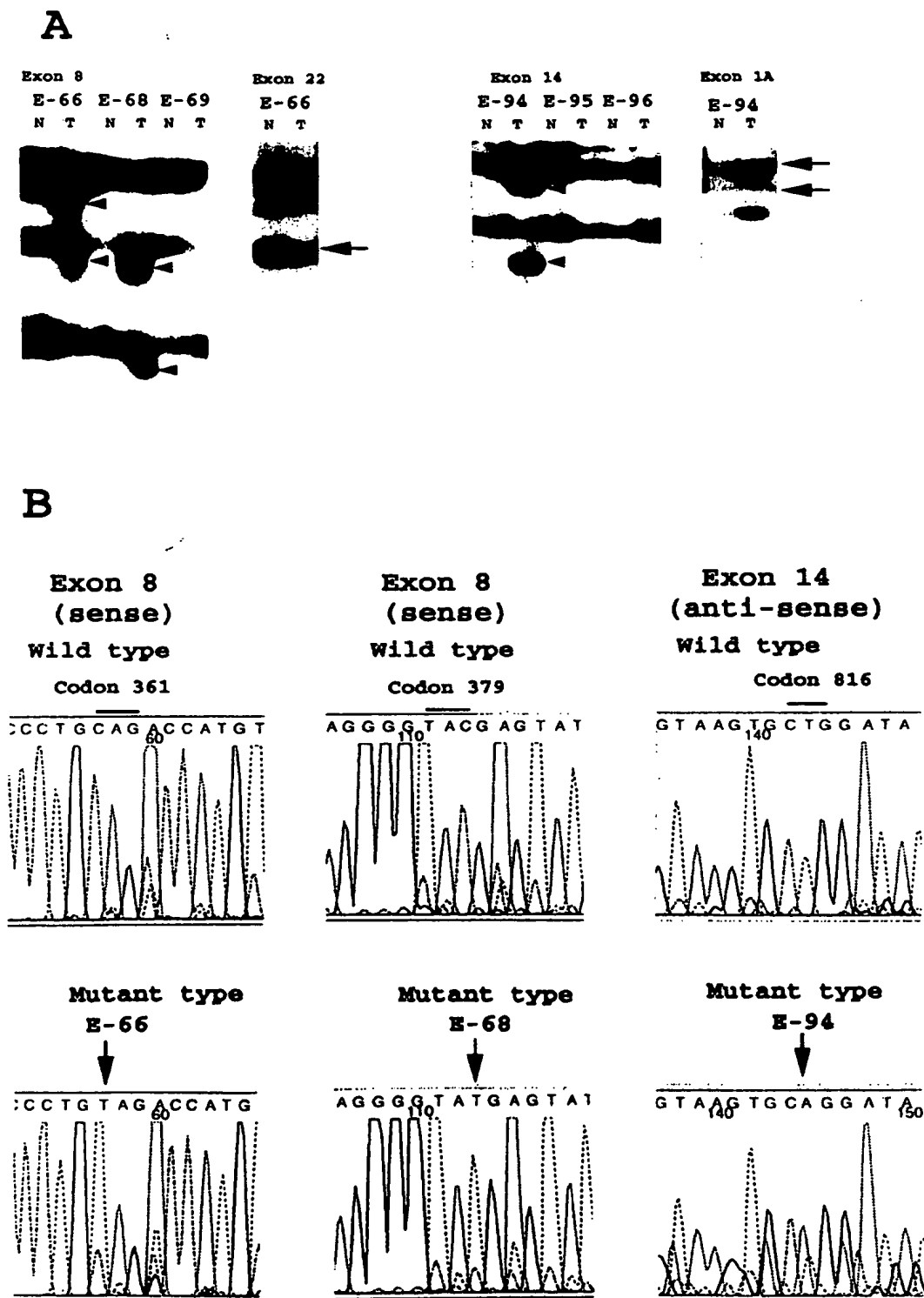


Figure 1. A: PCR-SSCP analysis of the *PTCH* gene. Mobility shifts (arrowheads) are detected in cases E-66 and E-68 (exon 8) and E-94 (exon 14). Presence of LOH in cases E-66 (exon 22) and E-94 (exon 1A) is indicated by arrows. N = normal DNA, T = tumor DNA. B: Sequencing histograms of exons 8 and 14 in the *PTCH* gene. A nonsense mutation at codon 361 (case E-66), a silent mutation at codon 379 (case E-68), and a missense mutation at codon 816 (case E-98) are indicated by arrows.

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Filed: October 9, 2003
For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit I

Lu X et al, *International Journal of Gynecological Cancer* 2000;10:452-458.

Loss of heterozygosity among tumor suppressor genes in invasive and *in situ* carcinoma of the uterine cervix

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Abstract. Lu X, Nikaido T, Toki T, Zhai Y-L, Kita N, Konishi I, Fujii S. Loss of heterozygosity among tumor suppressor genes in invasive and *in situ* carcinoma of the uterine cervix. *Int J Gynecol Cancer* 2000;10:452-458.

The aim of the present study was to further clarify the histogenesis of cervical carcinoma by investigating loss of heterozygosity (LOH) among a number of tumor suppressor genes in invasive and *in situ* carcinoma of the cervix. Materials consisted of 16 *in situ* and 29 invasive carcinomas (16 squamous cell carcinomas, nine adenocarcinomas, and four adenosquamous carcinomas). DNA samples were collected by microdissection from ordinary formalin-fixed, paraffin-embedded tissues, both from the lesions and from normal tissues. LOH was analyzed using eight DNA polymorphic tumor suppressor markers. Of the 16 cases of carcinoma *in situ*, three cases exhibited LOH at one locus. Of the 29 cases of invasive carcinomas, six cases exhibited LOH at two loci and nine cases exhibited LOH at one locus. Overall, LOH was found more frequently in invasive carcinomas than in *in situ* carcinomas. LOH was most frequently detected at the PTCH (*Drosophila* patched gene) locus. There was no significant correlation between LOH at a specific site and either histologic subtype or clinical stage. These results suggest that LOH might already occur in a fraction of preinvasive squamous lesions and that accumulation of LOH may in part play a role in carcinogenesis of the cervix.

KEYWORDS: carcinoma *in situ*, cervical carcinoma, loss of heterozygosity, tumor suppressor gene.

The carcinogenesis of human malignant tumors requires multiple steps involving the accumulation of several genetic alterations⁽¹⁾. Indeed, multiple genetic changes, especially involving tumor suppressor genes, are associated with the development of a variety of human cancers⁽¹⁻³⁾. However, the actual development from *in situ* lesion to invasive carcinoma is not fully understood in most human cancers.

The pathophysiology of cervical neoplasia is one of the best understood among human malignancies, and has a defined preinvasive lesion. For instance, (squa-

mous cell carcinoma develops from squamous intraepithelial lesion (SIL), cervical intraepithelial neoplasia (CIN), or cervical dysplasia/carcinoma *in situ* (CIS), while adenocarcinoma often develops from adenocarcinoma *in situ*⁽⁴⁾. It is also well known that human papillomavirus (HPV) infection plays an important role in the development of cervical carcinomas, both squamous cell carcinoma and adenocarcinoma. High-risk HPVs, such as HPVs 16 and 18, have been demonstrated in SIL, invasive squamous carcinomas, adenocarcinoma *in situ*, and invasive adenocarcinomas⁽⁵⁾. The oncogenic potential of high-risk HPVs appears to be mediated by the HPV E6 and E7 proteins. In fact, the HPV E6 protein encoded by high-risk HPVs binds p53 and mediates its degradation⁽⁶⁾, while

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the HPV E7 protein, also encoded by high-risk HPVs, binds retinoblastoma gene (RB) protein and mediates its degradation⁽⁷⁾. However, previous studies have indicated that HPV infection alone does not lead to the development of a malignancy in the cervix⁽⁸⁾. Thus, certain genetic alterations, in addition to HPV infection, seem to be involved in the tumorigenesis of cervical carcinomas.

Although normal cells are heterozygous at many loci, malignant tumors are often homozygous at the same loci (loss of heterozygosity [LOH]). That is, the tumor DNA contains alleles from only one of the two chromosomes. LOH occurs by structural deletion of normal genes or loss of the chromosome carrying normal genes. Chromosomal analysis using polymorphic DNA markers that distinguish different alleles has revealed that LOH occurs in many chromosome regions in a variety of human malignancies. In particular, genetic alterations (point mutations and deletions) of tumor suppressors result in their inactivation or loss of function, and play an important role in the development of malignant tumors⁽²⁾. In cervical carcinomas, LOH has been reported to occur as allelic losses on different chromosome arms, a particular focus of attention being deletion in chromosome 3^(9–13). One or more tumor suppressor genes present in these regions are suspected of being involved in the carcinogenesis of cervical malignancies. LOH has been identified in cervical carcinoma in the TP53 and RB1 genes⁽¹⁴⁾, both of which are degraded by high-risk HPVs. LOH has also been identified on chromosome 3 in preinvasive lesions^(13,15–17). These studies seem to provide evidence that a number of genetic alterations may be involved in the development of cervical carcinomas. However, the frequency of LOH on chromosome 3 is considerably lower in CIN than in invasive carcinomas, and very few studies have examined LOH among tumor suppressor genes located on other chromosomes. Therefore, as a way of studying LOH in a variety of tumor suppressor genes in invasive and *in situ* cervical carcinomas, we set out to examine its occurrence using microsatellite markers of eight representative tumor suppressor foci in these cervical lesions that were available as microsatellite markers in the region of known tumor suppressor gene.

Materials and methods

Case selection

Sixteen cases of CIS (high grade SIL/CIN3), 16 cases of invasive squamous cell carcinoma, nine cases of pure

adenocarcinoma, and four cases of adenosquamous cell carcinoma were studied. According to the FIGO classification⁽¹⁸⁾, 18 cases were at stage IB1, six at stage IIB, and five at stage III. Forty tissue samples were obtained from hysterectomy specimens and five by biopsy. Histopathologic diagnoses were confirmed by reviewing hematoxylin and eosin-stained slides for all the cases. All the tissues had been fixed in 10% phosphate-buffered formalin and embedded in paraffin. The use of these histological specimens was approved by the Clinical Research Ethics Committee of Shinshu University.

Microdissection and DNA extraction

Two trained gynecological pathologists (T.T. and S.F.) determined the tissues to be microdissected for LOH analysis. Using a razor, the tissues were scraped from definite *in situ* or invasive carcinoma lesions. This was done from consecutive tissue sections under a microscope, with a hematoxylin and eosin-stained slide used as a template. In cases of adenosquamous carcinoma, both glandular and squamous lesions were scraped for the LOH analysis. The corresponding control tissues were obtained from uninvolved myometrium or normal fallopian tube in each case. For DNA extraction, seven or eight 8- μ m-thick consecutive tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. They were incubated for 16–24 h at 55°C in a digestion buffer (2 mg/ml proteinase K and 0.5% Tween 20)⁽¹⁹⁾, followed by a series of phenol/chloroform extractions interrupted by an RNase digestion. The DNA was finally precipitated in cold ethanol with sodium acetate, and resuspended in Tris-EDTA buffer, pH 7.5.

LOH analysis

The sequences of eight markers (TP53, RB1, DCC, NM23, WT1, P16, DPC4, and PTCH) that were available as microsatellite markers in the region of known tumor suppressor gene, were obtained from previous reports^(20,21) or from the Genome Data Base ([HTTP://www.gdb.org/](http://www.gdb.org/)). The chromosomal locations and the sequences of these eight markers are listed in Table 1. The oligonucleotides were labeled fluorescently with Texas Red (5' oligonucleotide Texas Red labeling kit; Amersham Life Science, CA, USA). The PCR amplifications were performed in a volume of 10 μ l and involved the use of 1x PCR buffer, 1.25 mM MgCl₂, 200 μ M of each dNTP, 1% deionized formamide, 50 ng DNA, 0.25 U TaKaRa Taq polymerase (Takara, Ohtsu,

Table 1. Microsatellite markers used for the LOH analysis

Microsatellite markers	Localization	Primer sequences
p16	9p21	5'GGCTTTCTCTTTTGTCTC 3'GGAATAAATCAGGCTACCAGG
PTCH	9q22.3	5'CATTGGTTTGAATCGAACC 3'AGCTATTTTGGGGGCTGAG
WT1	11p13	5'CTTACTGGGTGAGGAAATCC 3'ACAGTAATTTCAAGCAACGG
RB1	13q14.2	5'CTCCTCCCTACTTACTTGT 3'AATTAACAAGGTGTGGTGGTACACG
TP53	17p13.1	5'ACTGCCACTCCTTGCCCCATT 3'AGGGATACTATTGAGCCCGAGGTG
NM23	17q21.3	5'TTGACCGGGGTAGAGAACTC 3'TCTCAGTACTTCCCGTGACC
DPC4	18q21.1	5'TGGGGTGTGTACCAGCATC 3'TGGCTTTCAATGTCAGAAAGG
DCC	18q21.3	5'GATGACATTTCCCTCTAG 3'GTGGTTATTGCCTTGAAAAG

LOH indicates loss of heterozygosity.

Japan), and 2 pmol of fluorescently labeled primer. Fifty nanograms of genomic DNA underwent thermal cycling as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min each, 58°C or 62°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 2 min. Amplified products were diluted 1:1 with a stop-solution containing 95% formamide, then denatured at 94°C for 3 min. Samples were loaded on a 6% polyacrylamide/7.7 M urea denaturing gel containing 30% deionized formamide, then run for 10–15 h in an Automated Sequencer (Hitachi, Ibaragi, Japan) at a constant power of 30 W. VISTRA DNA Size Standards (50–500 base pairs) (Amersham) were used to provide DNA size-markers. The data were collected automatically and analyzed using FRAGLYS v2 software (Hitachi). To judge the presence or absence of LOH, we used two criteria, namely band instance and allele ratio. The normal DNA samples were used to determine whether the sample was homozygous (one band or one peak only is seen) or heterozygous (two bands or two peaks are seen). For a heterozygous sample, LOH was considered to be present when one of the two alleles was completely absent from the tumor DNA or when the intensity ratio was obviously different (see below) from that of the constitutional DNA. The intensity ratio of alleles was calculated for each pair of normal and tumor samples, and then the tumor ratio was divided by the normal ratio: T1 (Tumor 1):T2 (Tumor 2)/N1 (Normal 1):N2 (Normal 2).

An intensity ratio of less than 0.5 was considered to indicate the presence of LOH⁽²²⁾. Statistical analysis was performed using Fisher's exact probability test.

Results

PCR-LOH assay of tumor suppressor genes

It is difficult to amplify DNA from paraffin-embedded tissues, particularly when the amplified fragment is larger than 300 bp, perhaps due to DNA degradation. This limitation has prevented the use of large RFLP markers in established LOH from archival specimens. To address this problem, we used microsatellite markers with short variable numbers of tandem repeats and analyzed the amplification products on denaturing sequencing gels. These markers are reliable and highly informative. LOH was judged to be present when the tumor sample showed one allele loss by comparison with the normal tissue from the same patient (Fig. 1A) and/or when the intensity ratio was less than 0.5 (Fig. 1B). Figure 2 demonstrates examples of the PCR-LOH assay in the TP53, RB1, p16, and PTCH genes. An example of LOH in a case of squamous cell carcinoma is shown in Figure 3. The informativity and the presence of LOH for all genes studied are summarized in Table 2.

Occurrence of LOH by histologic subtypes

Of the 16 cases of CIS, three cases exhibited LOH at one locus (PTCH, DCC, or RB1). Of the 29 cases of invasive carcinomas, six cases exhibited LOH at two loci, 11 cases at one locus, while no LOH was demonstrated in 12 cases. The frequency of LOH at different chromosomal loci is shown for each histologic subtype in Table 3. (Among all 45 cases examined, LOH was

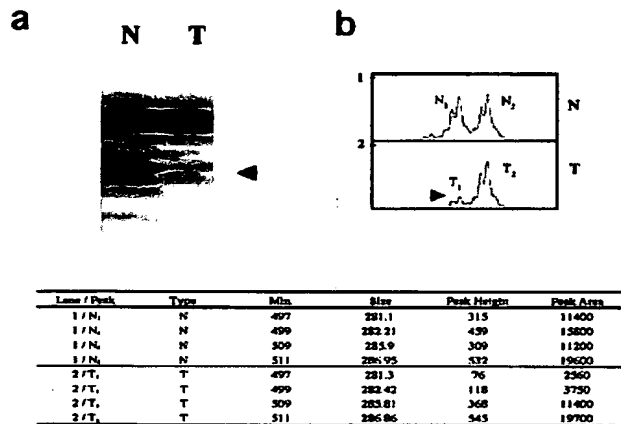


Fig. 1. Loss of heterozygosity analysis using band instance (a) and allele ratio (b). N indicates normal control; T, tumor. (a) Allelic loss is detected in tumor in the patient. (b) Electrophoretogram for a normal:tumor pair in which allelic loss is detectable in the tumor, the relative peak heights of the upper alleles are very similar in the normal (lane 1) and tumor (lane 2) DNA samples and the allele ratio (in this example) is 0.92 [(T₂/N₂), T₂ and N₂ are the area values of the shorter length allele]. By contrast, in the electrophoretogram for a normal:tumor pair; an indication of the loss of the smaller-sized allele in the tumor DNA (lane 2, T₁) can be seen by the decrease in the height of this peak. Calculation of the allele ratio in the latter example gives a value of 0.21, which confirms the loss. The data below each electrophoretogram show the type, mines, size, height, and area of each peak. Arrows indicate allelic loss in tumor DNA.

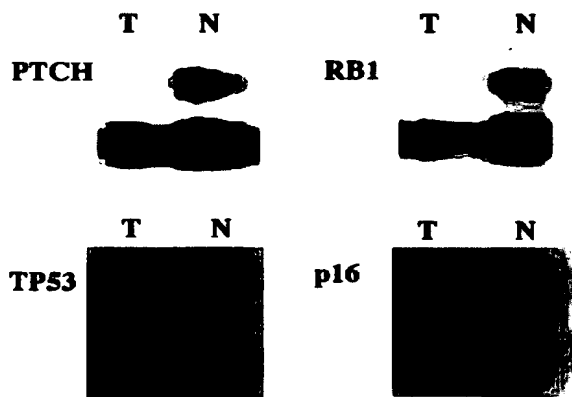


Fig. 2. Loss of heterozygosity at PTCH, RB1, TP53, and p16 in paired normal and tumor tissues. T indicates tumor tissue; N, normal tissue. Note that the tumor sample shows one allele loss for PTCH and RB1, and that the intensity ratio is less than 0.5 for TP53 and p16.

(detected most frequently at the PTCH locus) (eight cases), followed by at TP53 or RB1 locus (five cases each). Overall, LOH (at any locus) was found more frequently in invasive carcinomas than in *in situ* carcinomas ($P = 0.013$). There was no significant correla-



Fig. 3. Loss of heterozygosity detected at two loci in a case of squamous cell carcinoma. T indicates tumor tissue; N, normal tissue.

tion between LOH at a specific site and histologic subtype or clinical stage in invasive carcinomas.

Discussion

In this study, we have demonstrated LOH in a number of tumor suppressor genes not only in invasive cervical carcinomas but also in preinvasive intraepithelial lesions. Among the markers we tested, LOH was found at PTCH, DCC, and RB1 in preinvasive squamous lesions of the cervix, although the number of cases showing LOH was small. Guo *et al.* pointed out that chromosome 3p deletions were observed more frequently in CIN with coexisting cancer than in CIN without coexisting cancer⁽¹⁵⁾. The CIS (CIN III) lesions we studied were not associated with coexisting cancer, and LOH was infrequent among the markers tested. Thus, LOH may not be common in preinvasive lesions without coexisting invasive cancer, but LOH at a certain locus already occurs in a small fraction of preinvasive squamous lesions of the cervix.

In the present study, LOH at TP53 (whose protein is degraded by the E6 protein of high-risk HPVs⁽⁶⁾) was only detected in five cases of invasive carcinoma. Although allelotyping analysis of cervical carcinomas has revealed that about 20–40% of these tumors have allelic loss on chromosome 17p13.1 (which contains TP53)^(12,23), actual genetic alterations in TP53 (either point mutations or LOH) have been reported to be unusual in cervical carcinomas^(14,24). Conversely, in the present study, LOH at RB1 (whose protein is degraded by the E7 protein of high-risk HPVs⁽⁷⁾) was only detected in five cases of invasive carcinoma. Alterations in RB1 have been reported to be unusual in cervical carcinomas⁽¹⁴⁾. It is noteworthy that an associated high-risk HPV infection is present in approximately 80% of squamous carcinomas and 50% of adenocarcinomas of the cervix⁽⁵⁾. Thus, there is a discrepancy between the frequent association of cervical lesions with high-risk HPVs and the relatively infrequent p53 or RB gene alterations seen in these lesions. Searches for translation levels and function of

Table 2. Distribution of LOH in *in situ* and invasive carcinoma of the cervix

Case	Age	Diagnosis	p16	PTCH	WT1	RB1	TP53	NM23	DPC-4	DCC
1	25	CIS	-	-	-	-	-	-	-	-
2	26	CIS	-	-	-	-	-	-	-	-
3	31	CIS	-	-*	-	LOH	-	-	-	-
4	32	CIS	-	-	-	-	-	-	-	-
5	33	CIS	-	-*	-	-	-	-	-	-
6	35	CIS	-	-	-	-	-	-	-	-
7	36	CIS	-	-*	-	-	-	-	-	-
8	37	CIS	-	-	-	-	-	-	-	-
9	41	CIS	-	-	-*	-	-	-*	-	-
10	45	CIS	-	-	-	-	-	-	-	-
11	46	CIS	-	-	-	-*	-	-	-	-
12	47	CIS	-	-	-	-	-	-*	-	-
13	63	CIS	-	-	-*	-	-	-	-*	LOH
14	67	CIS	-	-*	-	-	-	-	-	-
15	71	CIS	-	LOH	-	-	-	-	-	-
16	71	CIS	-	-	-	-	-	-*	-*	-
17	29	SCC	-	-*	-*	-	LOH	-	-	-
18	30	SCC	-	-	-	-	-	-	-	-
19	32	SCC	-	LOH	-	LOH	-	-	-	-
20	44	SCC	-	-	-	-	-	-*	LOH	-
21	45	SCC	-	-	-	-	LOH	-	-	-
22	51	SCC	-	-	-	LOH	-	-	-	-
23	52	SCC	LOH	-	-	-	-	-*	-	-
24	52	SCC	-	-	-	-	-	-	-	-
25	55	SCC	-	-	-	-	-	-	-	-
26	58	SCC	-	-	-	-*	-	-	-	-
27	58	SCC	-	-	-	-	-	-	-	-
28	60	SCC	-	LOH	-	-*	-	-	LOH	-
29	62	SCC	-	LOH	-	-	-	-*	-	LOH
30	69	SCC	-	-	-	-	-	-	-	-
31	70	SCC	-	LOH	-	-	-	-	-	-
32	75	SCC	-	-	-	-	-	-	-	-
33	30	ADC	LOH	-	-	-	-	-	-	-
34	34	ADC	-	-*	-*	-	-	-	-*	-
35	36	ADC	-	LOH	-	-	LOH	-	-	-
36	37	ADC	-	-	-	-	-	-	-	-
37	44	ADC	-	-	-	-	LOH	-	-	-
38	47	ADC	-	-	-	-	-	-*	-	-
39	63	ADC	-	LOH	-	-	-	-	-	-
40	67	ADC	-	-*	-	-	-	-	-	-
41	71	ADC	-	-	-	LOH	-	-	-	-
42	41	ASC	-	-	LOH	-	LOH	-	-	-
43	43	ASC	-	-	LOH	-*	-	-*	-	-
44	43	ASC	-	-	-	-	-	-*	-	-
45	64	ASC	-	LOH	-	LOH	-	-	-	-

CIS indicates carcinoma *in situ*; SCC, squamous cell carcinoma; ADC, adenocarcinoma; ASC, adenosquamous carcinoma; LOH, loss of heterozygosity; -*, homozygous.

p53 and RB seem to be necessary. Other studies have also failed to demonstrate an evident correlation between LOH and HPV infection^(11,12,14,16). However, it is widely recognized that the inactivation of p53 and RB function occurs at the protein level through the activation of the E6 and E7 HPV proteins.

Thus, the correlation between HPV infection and alterations in p53 or RB1 in human cervical carcinomas has not been clearly confirmed by the *in vivo* evi-

dence. However, there are numerous mechanisms which can result in tumor suppressor gene inactivation together with LOH such as methylation, mutation, or altered expression. Investigation on these genetic alterations seems to be necessary.

Overall frequency of LOH (at any locus) was higher in invasive carcinomas than in *in situ* carcinomas, which appears to suggest that an accumulation of LOH may in part play a role in carcinogenesis of the

Table 3. Summary of occurrence of LOH in *in situ* and invasive carcinoma of the cervix

Histology	No. with any LOH	No. of LOH at particular loci						
		p16	PTCH	WT1	RB1	TP53	DPC4	DCC
CIS	3/16 (19%)	0	1	0	1	0	0	1
SCC	9/16 (56%)	1	4	0	2	2	2	1
ADC	5/9 (56%)	1	2	0	1	2	0	0
ASC	3/4 (75%)	0	1	2	1	1	0	0

CIS indicates carcinoma *in situ*; SCC, squamous cell carcinoma; ADC, adenocarcinoma; ASC, adenosquamous carcinoma; LOH, loss of heterozygosity.

cervix. However, no significant difference was found between frequency of LOH and clinical stage in invasive cases, a result consistent with a previous report⁽¹⁴⁾. Thus, the occurrence of LOH does not seem to correlate with disease progression in invasive carcinomas. However, the number of the cases studied so far is small, and further investigations will be required to elucidate the relationship between occurrence of LOH and clinical stage or disease progression.

(LOH was most frequently detected at the PTCH locus) in the present study. (The PTCH gene is a human homolog of the *Drosophila* patched gene. The patched protein has an important regulatory role in the hedgehog signaling pathway, a pathway that is critical for embryo segmentation and other steps in *Drosophila* development)^(25,26). Interestingly, frequent LOH at the PTCH locus has been reported in basal cell carcinoma of the skin, and it has been shown that PTCH acts as a tumor suppressor gene in such carcinomas^(26,27). Since the clinical implications of LOH at the PTCH locus are not completely understood, searches for LOH at the PTCH locus in other skin malignancies, especially in female vulvar carcinoma and vulvar intraepithelial neoplasia, might produce interesting data.

In summary, we found LOH in a variety of tumor suppressor genes in preinvasive squamous lesions of the cervix, as well as in invasive cervical carcinomas. Although the biological and clinical implications of LOH among tumor suppressor genes remain unknown, the occurrence of such genetic alterations further supports the involvement of tumor suppressor genes in the development of cervical malignancies.

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DECLARATION OF DR. SINAN TAS

Exhibit J

Berman DM et al, *Science* 2002;297:1559-1561.

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 Materials and Methods

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Medulloblastoma Growth Inhibition by Hedgehog Pathway Blockade

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Constitutive Hedgehog (Hh) pathway activity is associated with initiation of neoplasia, but its role in the continued growth of established tumors is unclear. Here, we investigate the therapeutic efficacy of the Hh pathway antagonist cyclopamine in preclinical models of medulloblastoma, the most common malignant brain tumor in children. Cyclopamine treatment of murine medulloblastoma cells blocked proliferation in vitro and induced changes in gene expression consistent with initiation of neuronal differentiation and loss of neuronal stem cell-like character. This compound also caused regression of murine tumor allografts in vivo and induced rapid death of cells from freshly resected human medulloblastomas, but not from other brain tumors, thus establishing a specific role for Hh pathway activity in medulloblastoma growth.

Signaling by the Hh family of secreted proteins was implicated initially in determination of embryonic cell fate, and more recently in maintenance of somatic stem cells and in specification of organ size. The latter role is illustrated in the developing cerebellum, where Hh signaling delays neuronal differentiation and induces proliferation of cerebellar granular neuronal precursors (CGNPs) (1–4). Medulloblastomas, which are aggressive childhood tumors of cerebellar origin, are associated with inappropriate Hh pathway activity (5–8). The activation of this pathway normally requires antagonism of the 12-transmembrane protein Patched (Ptc) by Hh ligand, thus releasing the seven-transmembrane protein Smoothened (Smo) for activation of target genes by the Cubitus interruptus/Gli (Ci/Gli) family of transcription factors (9–11).

Ligand-independent pathway activity in medulloblastoma is caused either by mutations that render Smo insensitive to regulation by Ptc or by mutational inactivation of *Ptc*. The transcription of *Ptc* is induced by Hh pathway activity, thus generating a negative feedback loop and serving as a convenient indicator of pathway activation.

We studied Hh pathway activity and function in cerebellar tumors from mice carrying a single mutant allele of the *Ptc* gene (*Ptc*^{+/−}) (5). Wild-type *Ptc* mRNA expression was never detected in pure tumor tissue, indicating a lack of functional *Ptc* gene product that should result in Hh pathway activation (see supporting online material, fig. S1). The frequency of medulloblastomas in *Ptc*^{+/−} mice was increased by a *p53* mutant background (12); loss of *p53* function also enabled propagation of these tumors in athymic mice as subcutaneous allografts that displayed diagnostic features of human medulloblastoma (Fig. 1, A and B; fig. S2) (13). From four such allografts independently originating in *Ptc*^{+/−} mice, cultured cell lines were derived that lacked *p53* function (Fig. 1C), retained a *Ptc*^{+/−} genotype (Fig. 1C), and displayed elevated levels of Hh pathway activity (see below). Treatment with the DNA demethylating agent 5-azacytidine (5-

azaC) restored *Ptc* mRNA expression in each cell line (Fig. 1D) and dramatically reduced activity from the *Ptc*-*LacZ* reporter (Fig. 1E). As the *LacZ* gene disrupts the targeted *Ptc* allele, thereby reporting *Ptc* transcription, the observed decrease in β -galactosidase levels indicates a reduction in pathway activity (5, 14). We confirmed the specificity of this effect by stably transfecting cells to express high levels of Gli1, which activates the Hh pathway downstream of Ptc. In such cells, 5-azaC did not block Hh pathway activity (Fig. 1E), despite restoration of *Ptc* mRNA expression (15). These results indicate that silencing by DNA methylation of the functional *Ptc* allele is the precipitating event in pathway activation and tumor initiation in these animals.

To test the role of Hh pathway activity in tumor growth, we used cyclopamine, a plant-derived pathway antagonist that acts at the level of Smo (14). Medulloblastoma-derived cell lines were cultured with cyclopamine or with tomatidine, another steroidal alkaloid with little effect on the Hh pathway (16). By 72 hours, cyclopamine treatment resulted in a 60 to 80% reduction in growth relative to tomatidine in cultures of all tumor-derived cell lines, whereas growth of fibroblast control cells was unaffected (Fig. 2A). Cyclopamine also completely and specifically abolished β -galactosidase activity in all murine medulloblastoma lines (Fig. 2B), demonstrating that the effect of cyclopamine on cell growth parallels the reduction in Hh pathway activity.

The effect of cyclopamine treatment on murine medulloblastoma cell growth is largely mediated by inhibition of cell proliferation, because culture of the PZp53^{MED1} allograft line with cyclopamine reduced DNA synthesis by 90%, as compared to tomatidine-treated cells (Fig. 2C), with only low levels of apoptosis (less than 1% of cells) (15). This effect depends on specific pathway inhibition, because PZp53^{MED1} cells engineered to overexpress Gli1 retained high levels of β -galactosidase activity (15) and cell growth (fig. S3A) upon treatment with cyclopamine.

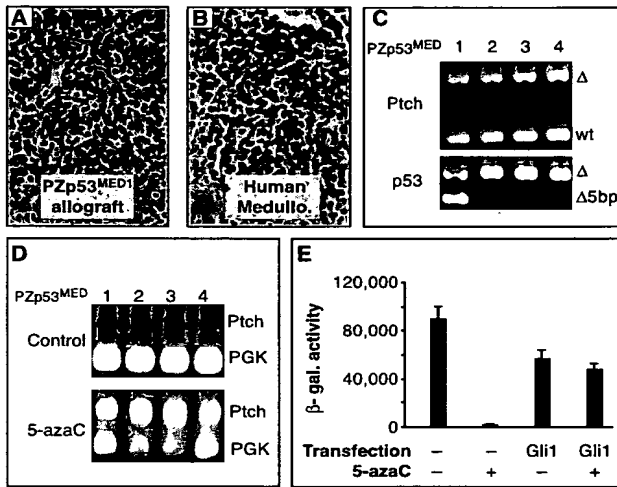
To determine the effect of Hh pathway inhibition on cellular pathways that regulate proliferation or differentiation, we examined the expression of cell cycle components and cerebellar neuronal differentiation markers in control and cyclopamine-treated PZp53^{MED1} cells. Cyclins D1, D2, E1, and hyperphosphorylated Rb,

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Fig. 1. Epigenetic basis of Hh pathway activation in a mouse medulloblastoma model. (A) Photomicrograph of PZp53^{MED1} allograft histology (hematoxylin and eosin stain) shows sheets of primitive round, basophilic (blue) cells with large, variably shaped nuclei and scant cytoplasm. Magnification is 200X. The morphology is similar to that shown in (B), which is human medulloblastoma (at the same magnification) with moderate anaplasia (abnormal nuclear morphology). (C) Genotypes of four medulloblastoma allograft lines arising in offspring of *Ptch*^{+/-} *p53*^{+/-} mice. Three lines were homozygous for the targeted *p53* allele (Δ) and a fourth (line 1) was heterozygous for the targeted *p53* allele and for an allele carrying a 5-bp deletion (Δ 5bp). (D) Multiplex reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA from the same four cell lines with primers against *Ptch* and phosphoglycerate kinase (PGK) shows absence of detectable *Ptch* mRNA and presence of PGK mRNA (positive control) in control cultures treated with ethanol vehicle. Epigenetic inactivation of *Ptch* is indicated by expression of *Ptch* mRNA in all four cell lines after culture in the presence of the demethylating agent 5-azacytidine (5-azaC). (E) β -galactosidase (β -gal.) activity from the *Ptch*-LacZ reporter is abolished in untransfected PZp53^{MED1} cells treated with 5-azaC, whereas β -gal. activity in PZp53^{MED1} cells stably transfected with Gli1 is unaffected by 5-azaC. Error bars represent standard error of the mean (SEM) for triplicate cultures.



which are induced by Shh in CGNPs and promote transit through prereplicative (G₁) cell cycle checkpoints (4), are also expressed in tomatidine-treated PZp53^{MED1} cells but are suppressed upon treatment with cyclopamine (Fig. 2D; fig S3B). In addition to down-regulation of a proliferative program, cyclopamine treatment appears to initiate differentiation in these cells, as indicated by reduced expression of the neurofilament nestin, a neuronal stem cell marker, and of the bHLH transcription factor Math1, a marker of proliferating CGNPs (17). Furthermore, cyclopamine treatment reduced expression of N-, c-, and L-myc (Fig. 2D, fig S3C) (18) and increased expression of NeuroD, a marker of postmitotic cerebellar granular neurons (Fig. 2D) (19). Morphological neuronal maturation was not observed in vitro, however, indicating that Hh pathway blockade only partially advances differentiation.

We further investigated cyclopamine effects in vivo and found by the 7th day of treatment that subcutaneous injections of the highest cyclopamine dose (1.25 mg/day; ~50 mg/kg) had abolished β -galactosidase activity (Fig. 3A) and growth (Fig. 3B) of medulloblastoma allografts propagated in nude mice. In a second experiment, allograft tumors from Gli-transfected cells (*n* = 7) grew despite cyclopamine treatment (Fig. 3C), whereas tumors from untransfected cells (*n* = 4) decreased in volume. Microscopic histologic analysis further demonstrated the complete disappearance of two of these tumors and a dramatic reduction in cell proliferation in the two that remained (fig. S4). These results demonstrate that cyclopamine can induce tumor regression by specific effects on the Hh pathway. No adverse effects were noted in cyclopamine-treated animals.

To explore the potential therapeutic utility of Hh pathway blockade, cells from freshly resected human medulloblastomas were cultured with KAAD-cyclopamine, a potent derivative of cyclopamine (14). Tumor cells initially displayed higher levels of *Ptch* mRNA than did normal cerebellum, indicating elevated Hh pathway activity, and this activity was reduced by treatment with either drug (fig. S5, A and B). Treatment with KAAD-cyclopamine also induced a significant decrease in cell viability within 48 hours (Fig. 4A); cell viability continued to decline after prolonged exposure (Fig. 4B), and fewer than 0.1% of cyclopamine-treated cells survived, after 1 week, as compared to typically greater than 10% of vehicle-treated controls. The effects of cyclopamine were similar to those of KAAD-cyclopamine (Fig. 4C) at pathway-inhibitory doses (14). Primary cultures from a glioblastoma and from an ependymoma, in contrast, failed to respond to cyclopamine (Fig. 4A). The dramatic reduction in cell viability of primary cultures from medulloblastomas, but not from other types of brain tumors, and the unaltered growth of embryonic fibroblasts (Fig. 2A) further suggests

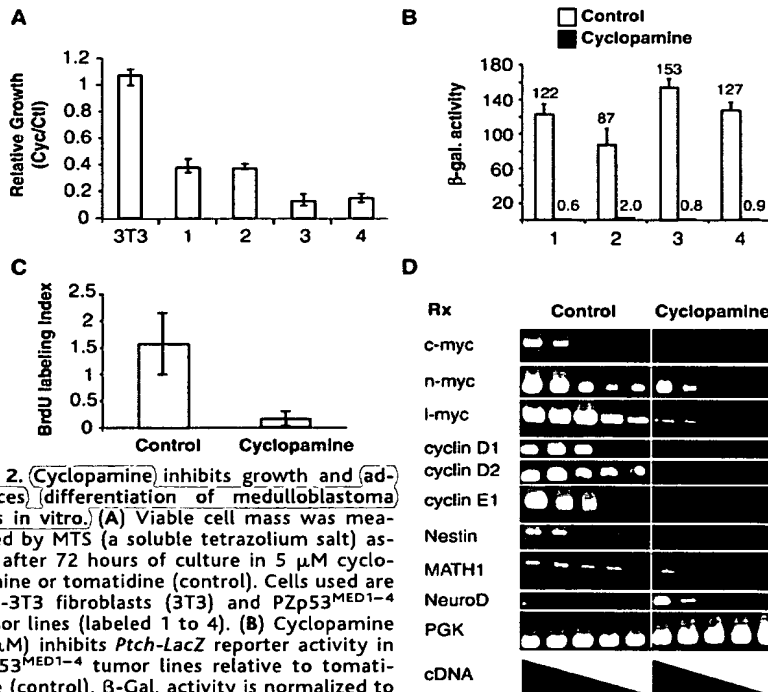


Fig. 2. Cyclopamine inhibits growth and advances differentiation of medulloblastoma cells in vitro. (A) Viable cell mass was measured by MTS (a soluble tetrazolium salt) assay after 72 hours of culture in 5 μ M cyclopamine or tomatidine (control). Cells used are NIH-3T3 fibroblasts (3T3) and PZp53^{MED1-4} tumor lines (labeled 1 to 4). (B) Cyclopamine (5 μ M) inhibits *Ptch*-LacZ reporter activity in PZp53^{MED1-4} tumor lines relative to tomatidine (control). β -Gal. activity is normalized to viable cell mass. (C) BrdU labeling indices for PZp53^{MED1} cells cultured with 5 μ M cyclopamine or tomatidine (control). (D) Semiquantitative RT-PCR analysis showing mRNA expression in PZp53^{MED1} cells cultured in tomatidine (control) or cyclopamine for 72 hours. cDNA was synthesized from 1 μ g of RNA from each culture. PCR was carried out on undiluted cDNA and on fourfold serial dilutions of cDNA, as indicated by the descending wedge.

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that cyclopamine specifically affects medulloblastomas and is not generally cytotoxic.

In contrast to reports suggesting that partial loss of *Ptch* function suffices to initiate tumor formation (20, 21), we found that medulloblastomas arising in *Ptch*^{+/−} mice lack detectable *Ptch* mRNA. The absence of *Ptch* mRNA and consequent pathway activation, apparently caused by DNA methylation of the normal *Ptch* allele, is critical not only for initiation but also for growth of the tumor, because pathway suppression by reactivation of the methylated *Ptch* allele or by cyclopamine treatment blocks growth. Loss of *p53* function in murine medulloblastomas appears to favor allograft and cell line growth, perhaps by compromising the ability of cells to undergo apoptosis (22). Human *p53* mutations are undetectable in nearly all (92 to 99%) sporadic medulloblastomas (23), which may account for increased apoptosis in primary human medulloblastoma cells cultured with Hh pathway antagonists. In light of the relatively low frequency (~30%) of sporadic human medulloblastomas linked to mutational activation of the Hh pathway (24, 25), it is perhaps surprising that elevated Hh pathway activity appears to be characteristic of these tumors and is

required for their growth (Fig. 4; fig. S5). The basis for this requirement is not clear, but may relate to known Hh functions as a stem cell factor in other tissues (9, 26) and as a mitogen in developing cerebellum (1–4). Hh pathway activity thus may play a role in maintenance of the capacity of medulloblastoma tumor stem cells (10) to undergo self-renewal and in the proliferation of their progeny. This dual role would be consistent with the high-level expression of the neuronal stem cell marker nestin in murine and human tumors (fig. S3C) (27) and with the rapid cell death of almost all human medulloblastoma cells when cultured with Hh pathway antagonists. Whatever its biological basis, the general requirement for Hh pathway activity in medulloblastoma growth represents a potential therapeutic opportunity, because cyclopamine and other pathway antagonists can be administered in effective doses with no apparent detrimental effects in rodents and other mammals (28).

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Supporting Online Material
www.sciencemag.org/cgi/content/full/297/5586/1559/DC1
Materials and Methods
Figs. S1 to S5
Table S1

8 May 2002; accepted 25 July 2002

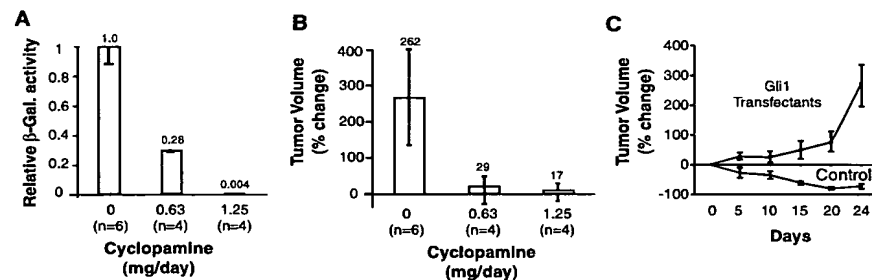
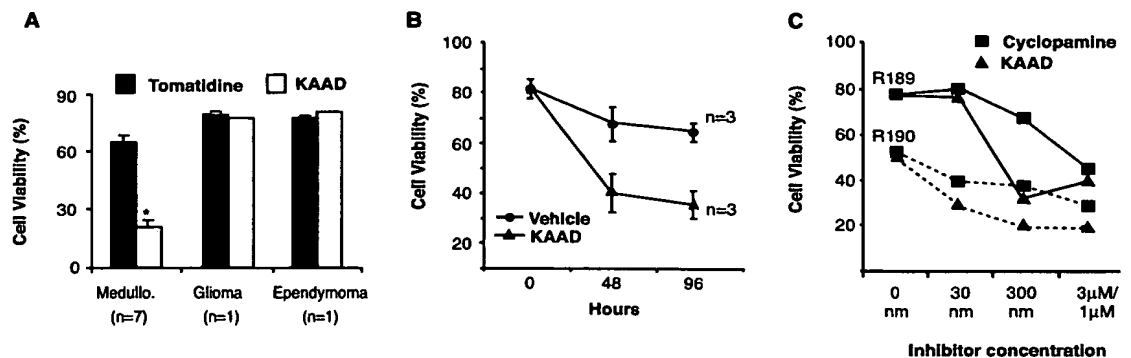


Fig. 3. Cyclopamine inhibits medulloblastoma growth through specific effects on the Hh pathway. (A) Dose-dependent *Ptch-LacZ* reporter inhibition in PZp53^{MED1} tumor allografts in tumor tissue from mice treated for 7 days with vehicle (0), or cyclopamine (0.63 or 1.25 mg/day). Error bars represent SEM. (B) Change in tumor allograft volume (%) in the same experiment. In this experiment, treatment was initiated after tumors reached an average size of 181 mm³. (C) Cyclopamine treatment induces regression of control-transfected tumors but not of tumors overexpressing the downstream Hh effector Gli1. Athymic mice bearing tumor allografts from control or Gli1-transfected PZp53^{MED1} cells were grown to ≤125mm³ before treatment with daily subcutaneous injections of cyclopamine (1.25 mg/day) for 24 days.

Fig. 4. Cyclopamine causes loss of viability of cultured tumor cells from primary human medulloblastomas. (A) Loss of cell viability in response to KAAD-cyclopamine (1 μM) 48 hours after surgical resection of tumor. Viability was assessed by absence of propidium iodide and annexin V-fluorescein isothiocyanate staining. Error bars represent standard error of the mean. (B) Loss of cell viability in response to KAAD-cyclopamine (1 μM) over time. (C) Dose response curves measuring loss of cell viability in response to increasing concentrations of cyclopamine (0 to 3 μM) and KAAD-cyclopamine (0 to 1 μM) in two medulloblastomas.



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For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit K

Thayer SP et al, *Nature* 2003;425:851-856.

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Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis

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Hedgehog signalling—an essential pathway during embryonic pancreatic development, the misregulation of which has been implicated in several forms of cancer—may also be an important mediator in human pancreatic carcinoma^{1–6}. Here we report that sonic hedgehog, a secreted hedgehog ligand, is abnormally expressed in pancreatic adenocarcinoma and its precursor lesions: pancreatic intraepithelial neoplasia (PanIN). Pancreata of Pdx-Shh mice (in which Shh is misexpressed in the pancreatic endoderm) develop abnormal tubular structures, a phenocopy of human PanIN-1 and -2. Moreover, these PanIN-like lesions also contain mutations in K-ras and overexpress HER-2/neu, which are genetic mutations found early in the progression of human pancreatic cancer. Furthermore, hedgehog signalling remains active in cell lines established from primary and metastatic pancreatic adenocarcinomas. Notably, inhibition of hedgehog

signalling by cyclopamine induced apoptosis and blocked proliferation in a subset of the pancreatic cancer cell lines both *in vitro* and *in vivo*. These data suggest that this pathway may have an early and critical role in the genesis of this cancer, and that maintenance of hedgehog signalling is important for aberrant proliferation and tumorigenesis.

Sonic hedgehog (SHH) is misexpressed in human adenocarcinoma and its precursor lesions. SHH expression was determined using *in situ* hybridization to detect SHH messenger RNA and immunohistochemistry (IHC) to detect the protein with an antibody directed against SHH⁹. Pancreatic tissues were obtained from 20 specimens resected for pancreatic cancer. Control pancreatic tissues with no evidence of abnormality or autolysis upon histological evaluation were obtained from autopsy specimens or from pancreatic resections for trauma. In normal adult human pancreata, no SHH was detected in the islets, acini or ductal epithelium (Fig. 1a). However, evaluation of pancreata from patients with adenocarcinoma reveals that SHH is aberrantly expressed in 70% of specimens. Normal ductal epithelium does not express detectable levels of SHH (Fig. 1b); however, as the ductal epithelium shows increasing degrees of atypia, PanIN-1 to -3 (Fig. 1c–e), a higher expression of SHH is observed. SHH expression is also detected in the malignant epithelium of adenocarcinoma samples (Fig. 1f). This expression pattern was also confirmed by our *in situ* hybridization for SHH mRNA (Supplementary Fig. 1).

Loss of regulation in this pathway has been implicated in several human cancers^{10,11}. Thus in order to determine the potential role of SHH misexpression in the adult human pancreas, pancreata from

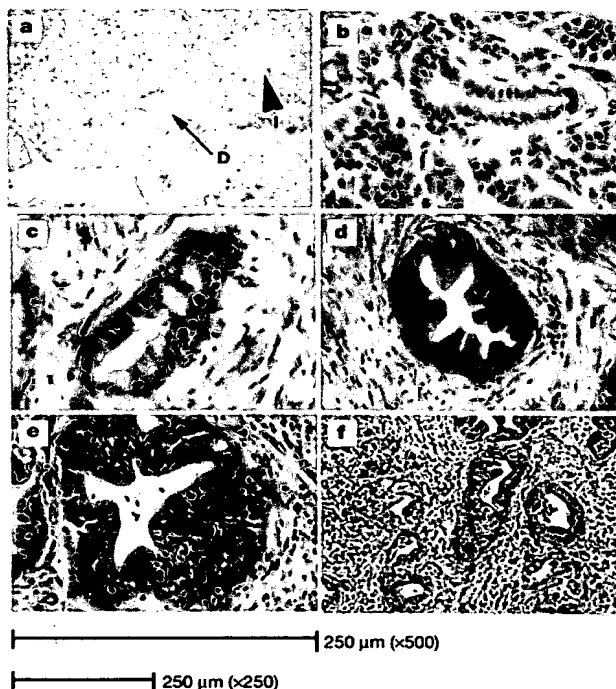


Figure 1 Immunohistochemical identification of SHH. **a**, Normal human pancreas. No specific staining for SHH protein was identified in acini, islets (I) or ducts (D) ($\times 125$ magnification). **b**, No SHH expression is detected in normal ductal epithelium ($\times 500$ magnification). **c**, PanIN-1 expresses minimal amounts of SHH ($\times 500$). **d**, PanIN-2 expresses moderate levels of SHH ($\times 250$). **e**, PanIN-3 ($\times 250$) and **f**, invasive cancer ($\times 125$): moderate to high levels of SHH.

letters to nature

transgenic mice (gift of H. Edlund) in which Shh misexpression was driven by the pancreatic-specific Pdx-1 promoter were histologically and immunohistochemically analysed.

A total of four pancreata from three-week-old Pdx-Shh mice

were histologically evaluated by a gastrointestinal pathologist (G.Y.L.). All four expressed a significant intestinal phenotype of the pancreatic epithelium. All four also exhibited abnormal tubular complex formations of the epithelium resembling pancreatic cancer precursor lesions (PanINs). These abnormal epithelial changes were characterized and classified according to the pathological classification scheme for human ductal lesions of the pancreas¹².

The transgenic pancreata display a mixed phenotype characterized by the transformation of acini into glandular structures lined by tall intestinal-type epithelium embedded in cellular spindle stroma. Focally, the metaplastic epithelium is composed of columnar cells with basally located nuclei and abundant supranuclear mucin (Fig. 2a). This phenotype is similar to human PanIN-1 (Fig. 2b), an early lesion in the stepwise sequence of pancreatic carcinogenesis. Furthermore, some glands are characterized by a lesser degree of mucin production but a higher grade of atypia, with nuclear disarray and stratification, mitosis and apoptosis (Fig. 2c), representing a higher degree of dysplastic change, similar to human PanIN-2 (Fig. 2d). According to the tumour progression model of

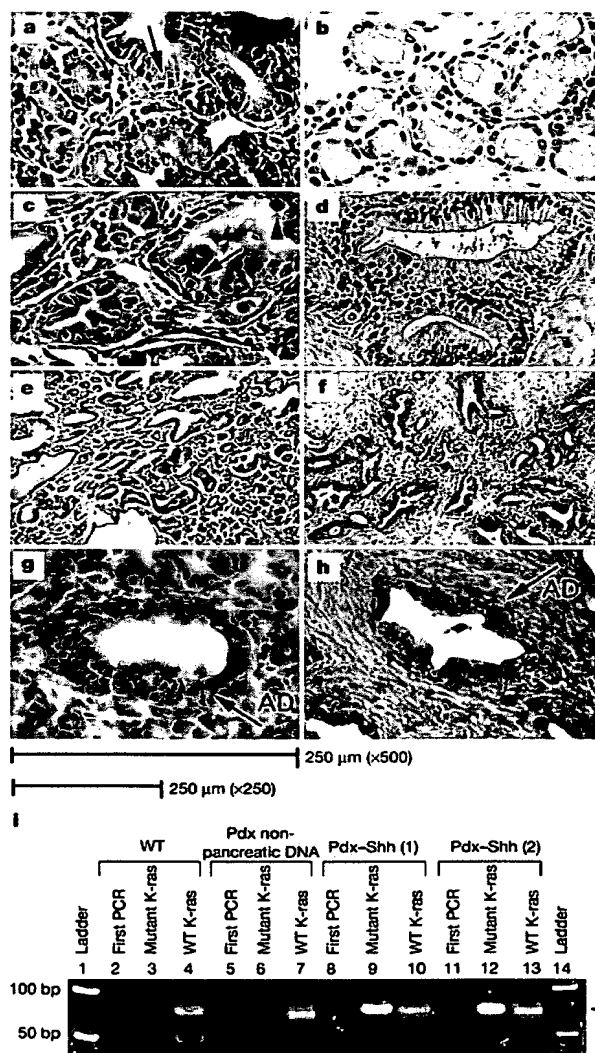


Figure 2 Histological comparison of human PanINs and Pdx-Shh mouse pancreata. **a**, Pdx-Shh pancreata display abnormal intestinal epithelial changes (arrow identifies abundant supranuclear mucin) that resemble human PanIN-1 (**b**) (x500). **c**, Pdx-Shh pancreata (x500) also exhibit a higher degree of atypia, with nuclear disarray and stratification (long arrow), mitosis (short arrow) and apoptosis (arrowhead), resembling human PanIN-2 (**d**) (x250). **e**, **f**, Low-magnification (x125) view of Pdx-Shh pancreata (**e**) and tubular complexes seen in human adenocarcinoma (**f**). **g**, Pdx-Shh mice (x500) and human PanINs (**h**) (x250) overexpress HER-2/neu, identified by a brown stain (AD; abnormal ductal epithelium). **i**, Mutant-allele-specific PCR amplification using mismatched primers (mutant K-ras) to detect codon 12 K-ras mutation. Wild-type primer (WT K-ras) is used as control. Lanes 1 and 14, ladder; 2, 5, 8, 11, first round of PCR (first PCR) reveals no detectable band, as expected. Wild-type mice express only wild-type K-ras (lanes 3, 4). Abnormal pancreatic epithelium of two Pdx-Shh mice expresses a TGT codon 12 mutation of K-ras (lanes 9, 12) and wild-type K-ras (lanes 10, 13), whereas the extrapancreatic tissues of Pdx-Shh mice outside the Shh expression domain express only wild-type K-ras (lanes 6, 7). The arrowhead identifies the 70-bp amplicon.

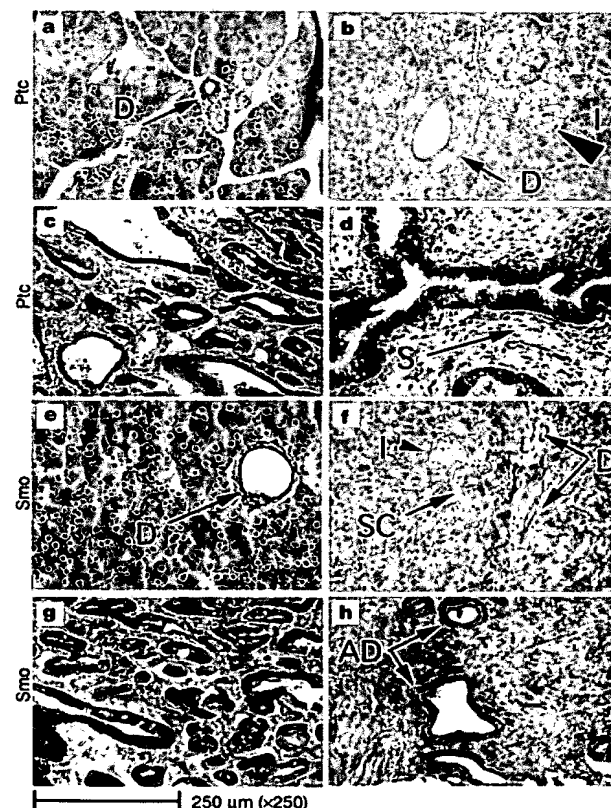


Figure 3 Hedgehog signalling pathway. **a-d**, Immunohistochemical identification of Ptc1 receptor. The Ptc1 receptor is not identified in normal acini, ducts or islets in either mouse (**a**) or human (**b**) pancreata. Ptc1 (brown stain) is markedly overexpressed in the abnormal pancreatic epithelium of both Pdx-Shh mice (**c**) and neoplastic human pancreata (**d**). In humans, PTC1 is also overexpressed throughout the reactive stroma (S) that surrounds the epithelium. **e-h**, Immunohistochemical identification of smoothened (Smo). Smoothened is not detected in normal duct epithelium (D) of normal mouse (**e**) or human (**f**) pancreata. Very infrequently Smo is detected in scattered cells (SC) in human pancreata; however, Smo (brown stain) is detected in the abnormal epithelium of Pdx-Shh mice (**g**) and neoplastic human pancreata (**h**) (arrows). The magnification is x250 for all images.

pancreatic carcinoma, these features are suggestive of an increasing neoplastic potential.

The histological progression model of pancreatic adenocarcinoma is also associated with progressive genetic alterations¹³. The transgenic mice pancreata, which histologically resemble PanIN-1 and -2, also express some of the early genetic alterations observed in the human progression model. Three pancreata overexpress HER-2/*neu* in the epithelium (Fig. 2g). In addition, preliminary data indicate that a codon 12 mutation in K-ras was present in two pancreata (Fig. 2i): changes noted to occur in human PanIN-1 and -2 lesions^{14,15}. Thus the Pdx-Shh mice develop abnormal pancreata with morphological and genetic changes that resemble human ductal PanIN.

Hedgehog (Hh) signalling is activated through binding of Shh to a membrane receptor, Ptc. This ligand-receptor interaction inhibits Ptc function, thereby allowing the de-repression of another transmembrane protein, smoothened (Smo), which ultimately leads to the activation of the Hh pathway and to the expression of Hh-specific genes, one of which is Ptc1 (ref. 16). Ptc1 expression (determined by IHC) is not detected in normal mouse or human pancreata (Fig. 3a, b). However, Ptc1 protein expression was detected throughout the abnormal epithelium in both Pdx-Shh mice (Fig. 3c) and human neoplastic pancreata (Fig. 3d). Furthermore, in human neoplastic pancreata, PTC1 was also detected in the

reactive mesenchymal cells that surround the abnormal epithelium (Fig. 3d).

Another member of the Hh pathway that is similarly overexpressed in both human neoplastic and mouse transgenic pancreata is Smo. In normal human and mouse pancreata Smo is not generally detected by IHC in the ducts or acini. Very infrequently Smo can be detected in an occasional duct cell or scattered acinar cell within the normal pancreas (Fig. 3f). However, Smo was overexpressed in the abnormal pancreatic epithelium found in Pdx-Shh mice (Fig. 3g), as well as in the epithelium of human adenocarcinoma and precursor lesions (Fig. 3h). The identification of Hh signalling members overexpressed in both mesenchyme and abnormal epithelium suggests that misexpression of Shh may be acting through both a paracrine and an autocrine mechanism to cause many of the morphological changes that characterize pancreatic cancer. To determine whether the HH pathway has an equally important role in the malignant biological behaviour of this tumour, we undertook work with pancreatic cancer cell lines.

Twenty-six human adenocarcinoma lines were screened for expression of HH signalling components. These cell lines were derived either from primary tumours (for example, the Panc series Panc 01.28 to 10.05 (ref. 17)) or from liver, lymph node and spleen metastases (CFPAC1, Hs 766T and SW 1990, respectively). All lines tested expressed two or more components of HH signalling,

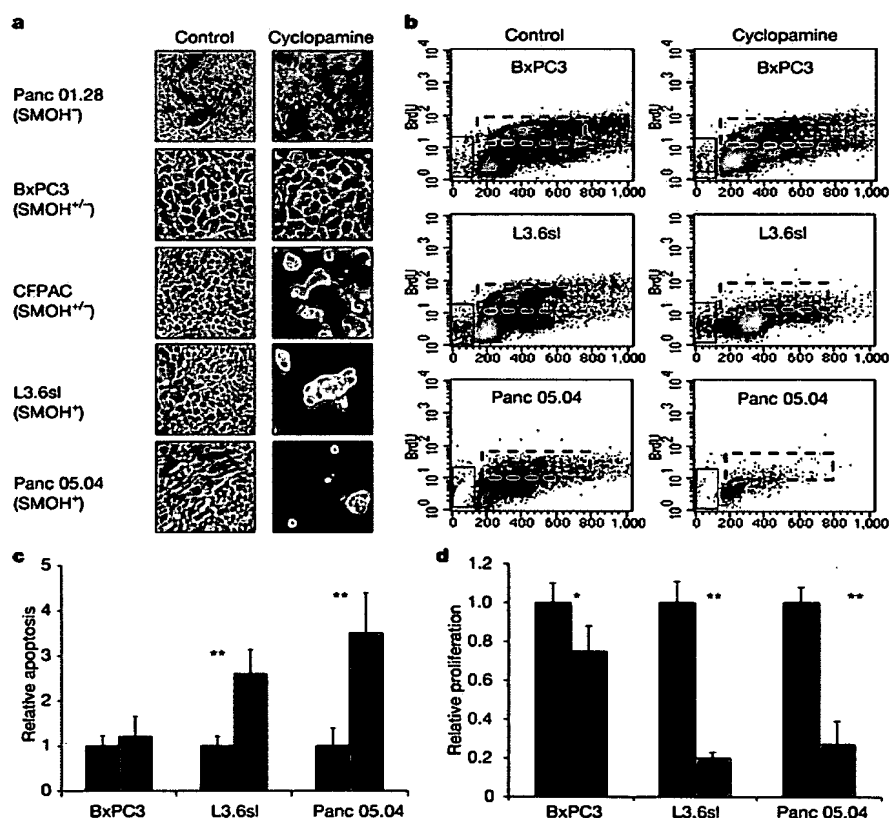


Figure 4 Effects of cyclopamine treatment on pancreatic adenocarcinoma cells. **a**, Cell cultures of five pancreatic adenocarcinoma cell lines untreated (control) or treated with 10 μ M cyclopamine for 7 days. **b**, Representative FACS histograms of three cell lines after BrdU incorporation ($n = 4$). The x axes show DNA content whereas the y axes show BrdU level; vertical boxes mark apoptotic cells; dashed horizontal boxes mark proliferating cells. **c**, Quantification of apoptotic cells measured in **b** ($n = 4$). To compare

treated and untreated cells, the number of apoptotic cells in control samples was adjusted to 1. **d**, Quantification of proliferating cells measured in **b** ($n = 4$). To compare treated and untreated cells, the number of proliferating cells in control samples was adjusted to 1. Error bars indicate standard deviation. Asterisk, $P < 0.05$; double asterisks, $P < 0.01$. SMOH⁻, no expression; SMOH^{+/+}, low level expression; SMOH⁺⁺, strong expression.

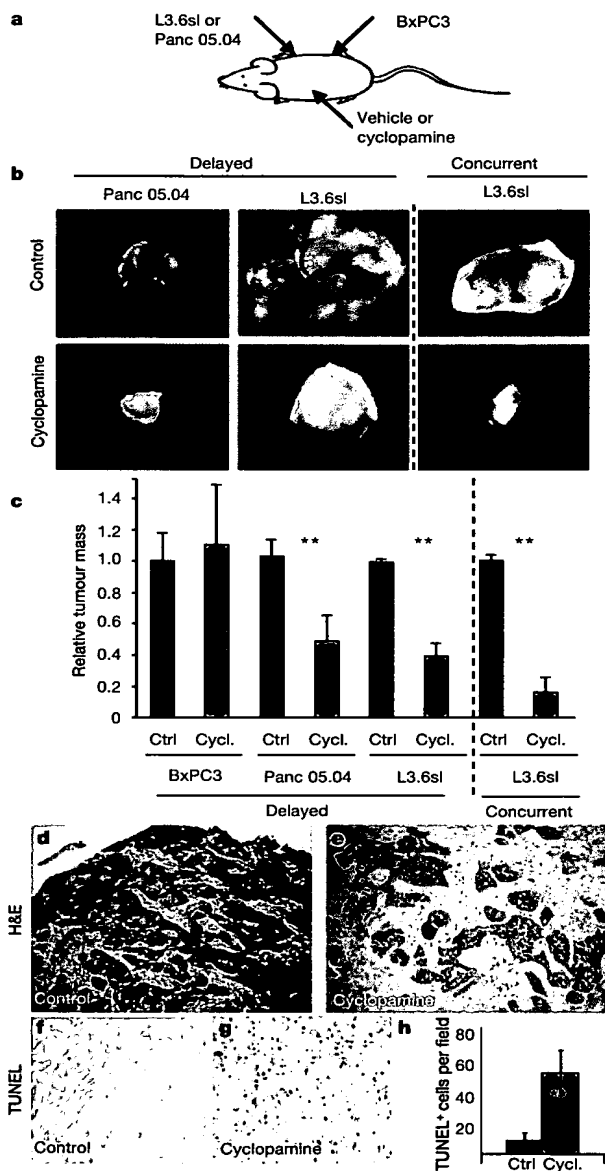


Figure 5 Cyclopamine treatment blocks tumour formation of human pancreatic adenocarcinoma cells after transplantation into nude mice. **a**, Schematic indicating sites of tumour cell and cyclopamine/vehicle injections. **b**, Isolated tumours derived from control or cyclopamine-treated L3.6sl and Panc 05.04 adenocarcinoma cells. Cyclopamine/vehicle injections were initiated either after palpable tumours had formed (delayed) or simultaneously with injection of tumour cells (concurrent). All pictures are shown at the same magnification. **c**, Weight of isolated tumours. Untreated control tumours of each cell line were adjusted to 1 to allow comparison of relative change in tumour mass. For delayed cyclopamine/vehicle injections, the values are: BxPC3, control 1 ($n = 6$), cyclopamine 1.1 ($n = 5$); Panc 05.04, control 1 ($n = 5$), cyclopamine 0.48 ($n = 4$); L3.6sl, control 1 ($n = 5$), cyclopamine 0.39 ($n = 4$). The values for concurrent cyclopamine/vehicle injections are: L3.6sl, control 1 ($n = 4$), cyclopamine 0.16 ($n = 4$). Error bars indicate standard deviation. Double asterisks, $P < 0.01$. **d–h**, Histological analysis of the effect of cyclopamine treatment on L3.6sl-derived tumours. **d**, **e**, Haematoxylin/eosin staining of sections through the peripheral tumour regions. **f**, **g**, TUNEL staining of apoptotic cells in control (**f**) and cyclopamine-treated (**g**) tumours. **h**, Quantification of TUNEL-positive cells in control (blue) and cyclopamine-treated (red) tumours. Error bars indicate s.e.m. Double asterisks, $P < 0.01$.

including *PTCH1*, *SMO*, *HIP* and *GLI1* (Supplementary Fig. 2). Thus, sustained HH signalling activity is detected in pancreatic adenocarcinoma cell lines isolated from both primary and metastatic tumours.

Cyclopamine, a steroidal alkaloid that inhibits Hh signalling through direct interaction with Smo^{18–20}, was used to test whether pancreatic adenocarcinoma cells require HH signalling for proliferation and survival. Initially five pancreatic cancer cell lines—BxPC3, Panc 01.28, CFPAC, L3.6sl and Panc 05.04—were qualitatively assessed. After 7 days of treatment, cyclopamine produced no change in cell density or morphology in control samples that included either untreated cells or cells treated with tomatidine, an inactive cyclopamine analogue. By contrast, a marked reduction in cell density was noted in cell lines CFPAC, L3.6sl and Panc 05.04. Morphologically, the majority of these cells detached from tissue culture plates during the treatment period, and the few remaining cells appeared aplastic (Fig. 4a, and data not shown).

Three representative pancreatic cancer cell lines—BxPC3-SMO^{low}, L3.6sl-SMO^{high} and Panc 05.04-SMO^{high}—were then used to quantitatively determine the effects of cyclopamine on proliferation and apoptosis by means of FACS analysis after 5-bromodeoxyuridine (BrdU) labelling. Cyclopamine did not induce apoptosis and only marginally reduced proliferation in the BxPC3-SMO^{low} control cell line. By contrast, apoptosis increased 2.5- to 3.5-fold accompanied by a 75–80% reduction in proliferation in the SMO^{high} cyclopamine-responsive cell lines L3.6sl and Panc 05.04 (Fig. 4b–d).

Our initial studies with five cell lines showed that only a subset of pancreatic cancer cell lines are susceptible to cyclopamine treatment. This could be the result of activating mutations of the HH pathway downstream of SMO, which should not be inhibited by this antagonist. To determine the percentage of cancer cell lines susceptible to cyclopamine treatment, we expanded our analysis to a total of 13 lines. Twelve of thirteen lines analysed expressed SMO; half of these (50%) responded to cyclopamine treatment. As predicted, growth of SMO-negative Panc 01.28 cells was not affected (Supplementary Fig. 3). Thus our data suggest that mutations in the HH pathway downstream of SMO might contribute to the aberrant proliferation of these cells. However, we cannot exclude the possibility that mutations in other, HH-independent, pathways regulate proliferation in cyclopamine-resistant pancreatic cancer cell lines.

To demonstrate that cyclopamine-induced inhibition of cancer cell proliferation is secondary to inactivation of the HH pathway, a Gli-luciferase reporter construct containing Gli-binding sites upstream of a TK minimal promoter²¹ was transfected into control and cyclopamine-responsive cell lines. GLI1 is a downstream transcription factor and transcriptional target of the Hh pathway²². All cell lines tested expressed GLI1 (Supplementary Fig. 2), and transfection of the Gli-reporter construct resulted in significant elevation of luciferase activity (Supplementary Fig. 4a), further indicating that Hh signalling is active in adenocarcinoma cells. Treatment of transfected cells with cyclopamine abolishes luciferase activity in responsive cell lines, whereas control cells display only an insignificant reduction in luciferase activity. Active HH signalling thus appears to be essential for cell survival and proliferation in a subset of cultured human pancreatic cancer cell lines.

As a more definitive test of the ability of cyclopamine to inhibit tumour cell proliferation, control (BxPC3) and cyclopamine-responsive cells (L3.6sl and Panc 05.04) were injected subcutaneously into immunocompromised nude mice (Fig. 5a). Two treatment models were used—concurrent or delayed treatments—in which cyclopamine was given either at the time of tumour implantation (concurrent) or when the subcutaneous tumours were palpable (delayed). Cyclopamine or carrier control was given for 7

days, at which time the tumours were excised and weighed. In the delayed treatment model, no difference in weight was noted between control and cyclopamine-treated BxPC3-SMO^{low} tumours (Fig. 5c). By contrast, a 50–60% decrease in tumour mass was observed in Panc 05.04- and L3.6sl-derived tumours, respectively (Fig. 5b, c)—an even more marked effect was noted in the concurrent treatment model, which revealed an 84% reduction in tumour mass of L3.6sl-derived tumours (Fig. 5b, c).

Histological analysis of L3.6sl-derived tumours (delayed treatment) revealed a compact mass of epithelial cells in untreated controls, whereas treated tumours appeared as loose epithelial cell aggregates with a larger amount of interspersed mesenchymal cells (Fig. 5d, e). TdT-mediated dUTP nick end labelling (TUNEL) assays demonstrated that cyclopamine treatment resulted in a sixfold increase in apoptotic cells compared with untreated controls (Fig. 5f–h). Therefore both *in vitro* and *in vivo* experiments reveal that HH signalling is important for proliferation and survival and thus may be an important mediator of the malignant biological behaviour of pancreatic adenocarcinomas in humans.

The observation that HH signalling has a critical and central role in pancreatic development and results in malignant transformation when mutated prompted us to ask about its potential role in pancreatic cancer^{16,23–25}. Our experiments lead us to believe that misregulation of HH signalling has a critical early role in the initiation and a later role in the maintenance of human pancreatic ductal adenocarcinoma, a hypothesis that is supported by findings in the accompanying paper³¹. Our evidence is based on comparative analysis of pancreata from Pdx1–Shh transgenic mice and of human adenocarcinoma. We find that HH signalling components are undetectable in normal human ductal epithelium but are strongly expressed in pancreatic precursor (PanIN) and invasive lesions. When Shh is misexpressed in Pdx–Shh mice, we detected the presence of abnormal pancreata with features that histologically, immunohistochemically and genetically resemble human PanIN lesions. Moreover, our analysis of human pancreatic cancer cell lines indicates that the HH signalling pathway is required for both cell division and the suppression of apoptosis. An important but unresolved question concerns the identity of the pancreatic cells that become transformed in response to increased HH signalling. Although pancreatic stem cells have not yet been isolated, results from studies in other tissues suggest that increased HH signalling in progenitor cells can elicit cancer formation. Future studies will be needed to elucidate the role of HH activity in stem-cell-mediated tissue regeneration and pancreatic adenocarcinomas.

Pancreatic cancer, the fourth highest cause of death from cancer in the United States, remains an incurable and rapidly lethal disease, with a 5-yr survival rate of less than 3%. Many factors contribute to this poor prognosis, but ultimately it is our lack of knowledge of the molecular determinants involved in the pathogenesis and progression of this tumour that has limited our ability to design effective treatments. Identification of a role for the HH pathway in the initiation and maintenance of pancreatic cancer suggests that this pathway may hold promise for new diagnostic and therapeutic approaches. □

Methods

Tissue preparation

Human tissue specimens were collected in accordance with IRB approval from the Massachusetts General Hospital. Archived specimens were fixed with formalin and paraffin-embedded according to standard institutional protocol. Wild type and Pdx–Shh mouse tissues were fixed in 4% paraformaldehyde, then paraffin-embedded as described above. Tissues were cut into 4–6-µm sections and applied to charged ProbeOn Plus slides (Fisher).

Transgenic mice

Initial pancreatic tissue (gift of H. Edlund) was in a B6/CBA background. Transient transgenic mice (founder mice) were generated by pronuclear injection of a *Not1/BamHI*

expression cassette containing a 4.5-kilobase (kb) *Not1–NaeI* genomic fragment of the Pdx promoter cloned in front of a 2.6-kb *XhoI* fragment of full-length rat *Shh* complementary DNA (vector also a gift of H. Edlund) into a B6/C3F1 background as previously described²⁶.

Immunohistochemistry

Single-antibody detection was accomplished as previously described²⁶, with the following protocol modifications: endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol for 10 min at room temperature. Antigen retrieval was achieved by boiling tissue in 0.01 M sodium citrate, pH 2.0, 6.0, or 8.0 for 10 min. Tissues were blocked first with 10% NGS in TBS for 40 min at room temperature, then with streptavidin followed by biotin for 20 min each at 37 °C. All primary antibodies were incubated overnight at 4 °C. Primary antibodies used were: Shh, Ptc1 and Smo (1:250; Santa Cruz). Secondary biotinylated antibodies (Vector Laboratories) were applied in 1:500 dilution for 1 h at room temperature. Detection of protein was visualized by brown pigmentation via standard DAB protocol. Slides were lightly counterstained with haematoxylin.

Detection of K-ras codon 12 mutation

The mutant-allele-specific amplification method was used for detection of K-ras codon 12 mutation as previously described^{27,28}. DNA was extracted from microdissected pancreata. Primers used for first-round PCR amplification for K-ras were K12 forward primer 5'-CGCGGCGGCTGAATGACTGA-3' and K12 reverse primer 5'-TCGTAGGGTCATCCTCATCC-3'. PCR conditions were: 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 0.5 min for 20 cycles. Then a second PCR was performed on 2.0 µl of the first reaction for an additional 40 cycles under similar conditions, using as the new upstream primer either a mismatched primer to amplify the specific mutant band (GGT to TGT), or a wild-type primer, producing a 72-base-pair (bp) mutant or 71-bp wild-type fragment. The second PCR downstream primer sequence is K12 R nest-in 5'-CCACAAAGTGATTCTGAATTA-3'. Mismatched upstream primer sequence is mutant K12 (TGT) 5'-TTGTGGTGGTTGGAGCTT-3'; and wild-type sequence is WT K12 5'-TGTGGTGGTTGGAGCTGG-3'. Amplicons were run on a 10% TBE polyacrylamide gel and stained with SYBR green (Molecular Probes).

Cell culture

Human pancreatic adenocarcinoma cell lines HPAC, SW1990, Mpanc-96, SU86.86, PL45, Panc 10.05, Panc 8.13 and Panc 2.03 were obtained from the American Tissue Culture Collection; cell lines MiaPaCa2, Panc-1, CFPAC1, HPAFII, Capan-2, AsPC1, Hs766T and BxPC3 were a gift from Schering Plough. The cell lines COLO357, L3.3, L3.6sl and L3.6pl were a gift from I. Fidler; cell lines Panc 3.07, Panc 5.04, Panc 2.13, Panc 6.03, Panc 4.21 and Panc 1.28 were a gift from E. Jaffee. BxPC3 and all the Panc cell lines were grown in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), L-glutamine and penicillin/streptomycin; medium for Panc cell lines was also supplemented with insulin–transferrin–selenium (Gibco). The CFPAC, Panc1, L3.6sl and L3.6pl cell lines were grown in DMEM without phenol red (CellGro), supplemented with 10% fetal calf serum. To test for cyclopamine responsiveness, cells were grown for 7 days in control medium containing tomatidine (Sigma) or DMSO alone or experimental medium containing cyclopamine (10 µM, Toronto Research Chemicals; the cyclopamine dose–response is shown in Supplementary Fig. 4b). We changed the medium every 2 days. Pictures showing cell morphology were taken with a Nikon Eclipse TE300.

BrdU incorporation assay

Cells were grown for 3 or 4 days in medium containing tomatidine (control, Sigma) or cyclopamine (10 µM, Toronto Research Chemicals). The medium was changed every 48 h. Cells were pulsed with 10 µM BrdU during the final 2 h of culture. BrdU was detected with a fluorescein isothiocyanate-conjugated anti-BrdU antibody (BD Biosciences); total DNA was stained with 7-AAD. FACS analysis was performed according to the BD Biosciences BrdU flow kit instruction manual. Cells in S phase were defined as a cell population that had incorporated BrdU, with DNA content comprising between 2N and 4N. According to the manual, apoptotic cells were defined as a subpopulation of G0/G1 cells with DNA content lower than the diploid amount.

Allograft treatment *in vivo*

Allograft treatment *in vivo* was performed according to ref. 19 with minor modifications. A total of 0.1 ml Hanks' balanced salt solution and matrigel (1:1) containing 2 × 10⁶ cells was injected subcutaneously into CD-1 nude mice. Tumours were grown for 4 days to a minimum volume of 125 mm³; treatment was initiated simultaneously for all subjects. Mice were injected subcutaneously with vector alone (triolein:ethanol 4:1 v/v) or a cyclopamine suspension (1.2 mg per mouse in triolein:ethanol 4:1 v/v) daily for 7 days. At the end of the treatment period, tumours were excised from mice, weighed and then fixed for 3 h at 4 °C with 4% paraformaldehyde, embedded in paraffin wax and sectioned (6 µm). Apoptotic cells were identified by TUNEL using recombinant Tdt (Invitrogen) as previously described²⁹. Sections were then counterstained with eosin. Eight ×20-magnified fields from regions corresponding to the exterior, middle and interior of two control and two cyclopamine-treated tumours were chosen at random. We counted the number of TUNEL-positive nuclei manually. Haematoxylin/eosin staining was done as previously described³⁰.

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Yip3 catalyses the dissociation of endosomal Rab-GDI complexes

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Human cells contain more than 60 small G proteins of the Rab family, which are localized to the surfaces of distinct membrane compartments and regulate transport vesicle formation, motility, docking and fusion^{1–3}. Prenylated Rabs also occur in the cytosol bound to GDI^{4,5} (guanine nucleotide dissociation inhibitor), which binds to Rabs in their inactive state. Prenyl Rab-GDI complexes contain all of the information necessary to direct Rab delivery onto distinct membrane compartments^{6–8}. The late endosomal, prenyl Rab9 binds GDI with very high affinity⁹, which led us to propose that there might be a 'GDI-displacement factor' to catalyse dissociation of Rab-GDI complexes and to enable transfer of Rabs from GDI onto membranes^{6,10}. Indeed, we have previously shown that endosomal membranes contain a proteinaceous factor that can act in this manner¹⁰. Here we show that the integral membrane protein, Yip3, acts catalytically to dissociate complexes of endosomal Rabs bound to GDI, and to deliver them onto membranes. We propose that the conserved Yip proteins serve as GDI-displacement factors for the targeting of Rab GTPases in eukaryotic cells.

Yip1p is an essential yeast protein that interacts with all yeast Rabs with varying affinities^{11,12}. Cells harbouring a temperature-sensitive allele of *yip1* display an accumulation of endoplasmic reticulum membranes and defects in secretion¹¹. Yeast and human cells contain a family of Yip-related proteins¹³, including Yif1, which interacts with yeast Rabs and Yip1p¹⁴. In mammalian cells, there are at least five Yip-related proteins; they appear to interact with each other and may function as distinct heterodimer pairs. Prenylated Rab acceptor (PRA-1) is the human Yip3 homologue: it interacts with multiple, prenylated Rabs^{15,16}, shows weak interaction with GDI¹⁷ and is localized to the Golgi¹⁸ and late endosomes (see Supplementary Information). The related PRA-2 is present in the endoplasmic reticulum; the carboxy termini of PRA proteins are responsible for their distinct localizations¹⁸.

His-tagged, human Yip3 was expressed in *Escherichia coli* and purified from the membranes after detergent extraction (Fig. 1a). Yip3 is predicted to have four transmembrane domains¹⁹, and the protein was highly susceptible to aggregation if detergent was limiting during purification. With adequate detergent, the protein chromatographed with an apparent mass of a monomer and could be obtained in highly purified form.

GDI binds tightly to Rab9⁹ and blocks release of bound GDP⁴. However, a GDI-displacement factor (GDF) facilitates the generation of free Rab9, which can then exchange bound GDP for ³⁵S-GTPγS (Fig. 1b)¹⁰. This method can detect a GDF, or alternatively, an unusual activity that could act on Rab-GDI complexes and stimulate that Rab's intrinsic nucleotide exchange rate (a 'GEF'). Purified, non-tagged, prenyl Rab9-GDI complexes were used to test whether purified human Yip3 could dissociate Rab-GDI complexes as monitored by an increase in the rate of ³⁵S-GTPγS binding. GDI release leads to prenyl-Rab aggregation; assays are carried out with just enough detergent (0.1% CHAPS) to stabilize a released Rab without destabilizing Rab-GDI complexes¹⁰. Once released from GDI, a Rab will exchange bound GDP for GTPγS and then be incapable of rebinding GDI.

Human Yip3 stimulated the rate of nucleotide binding to Rab9 when added to prenyl Rab9-GDI complexes (Fig. 1c). GDF activity

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In vivo inhibition of endogenous brain tumors through systemic interference of Hedgehog signaling in mice

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Abstract

The full spectrum of developmental potential includes normal as well as abnormal and disease states. We therefore subscribe to the idea that tumors derive from the operation of paradevelopmental programs that yield consistent and recognizable morphologies. Work in frogs and mice shows that Hedgehog (Hh)-Gli signaling controls stem cell lineages and that its deregulation leads to tumor formation. Moreover, human tumor cells require sustained Hh-Gli signaling for proliferation as cyclopamine, an alkaloid of the lily *Veratrum californicum* that blocks the Hh pathway, inhibits the growth of different tumor cells in vitro as well as in subcutaneous xenografts. However, the evidence that systemic treatment is an effective anti-cancer therapy is missing. Here we have used *Ptc1*^{+/-}; *p53*^{-/-} mice which develop medulloblastoma to test the ability of cyclopamine to inhibit endogenous tumor growth in vivo after tumor initiation through intraperitoneal delivery, which avoids the brain damage associated with direct injection. We find that systemic cyclopamine administration improves the health of *Ptc1*^{+/-}; *p53*^{-/-} animals. Analyses of the cerebella of cyclopamine-treated animals show a severe reduction in tumor size and a large decrease in the number of *Ptc1*-expressing cells, as a readout of cells with an active Hh-Gli pathway, as well as an impairment of their proliferative capacity, always in comparison with vehicle treated mice. Our data demonstrate that systemic treatment with cyclopamine inhibits tumor growth in the brain supporting its therapeutical value for human Hh-dependent tumors. They also demonstrate that even the complete loss of the well-known tumor suppressor p53 does not render the tumor independent of Hh pathway function.

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Keywords: Cancer; Tumor; Medulloblastoma; Brain; Mouse; Cyclopamine; Hedgehog; Gli

1. Introduction

Secreted glycoproteins of the Hedgehog (Hh) family participate in the development and homeostasis of many organs. Hh signal transduction in the target cells occurs through at least two membrane proteins, Smoothened (Smo) and Patched1 (Ptc1) (reviewed in Ingham and McMahon, 2001). In the absence of Hh, Ptc1 represses Smo. Hh inhibits Ptc1, allowing Smo to signal intracellularly and activate

the Gli zinc-finger transcription factors (reviewed in Ruiz i Altaba et al., 2002a). One of the genes that is positively controlled by the Hh pathway is *Ptc1* itself, thus generating a negative feedback loop and serving as a convenient indicator of pathway activation (Goodrich et al., 1996). Inappropriate activation of the Hh pathway has been associated with familial cancers in Gorlin's syndrome (Johnson et al., 1996; Hahn et al., 1996) as well as sporadic human cancers in tissues in which Hh signaling normally plays a role during patterning, growth or homeostasis, like skin (Dahmane et al., 1997; Nilsson et al., 2000; Xie et al., 1998), lung (Watkins et al., 2003), pancreas (Thayer et al., 2003), stomach (Berman et al., 2003), prostate (Sanchez et al., 2004; Karhadkar et al., 2004) and brain (Dahmane et al., 2001; Berman et al., 2002) (reviewed in Ruiz i Altaba et al., 2002b, 2004; Pasca di Magliano and Hebrok, 2003). This has led to the search and isolation of molecules with therapeutic potential capable of modulating the Hh

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pathway (Taipale et al., 2000; Chen et al., 2002; Frank-Kamenetsky et al., 2002; Williams et al., 2003). Such molecules include cyclopamine (Keeler and Binns, 1968; Keeler, 1978), an alkaloid that inhibits Hh signaling (Incardona et al., 1998; Cooper et al., 1998) by impairing the activity of Smo (Taipale et al., 2000) as well as siRNAs against GLI1 (Sanchez et al., 2004). Cyclopamine appears to be as effective as other compounds targeting Smo (Taipale et al., 2000; Chen et al., 2002; Frank-Kamenetsky et al., 2002; Williams et al., 2003) and inhibits tumor cell proliferation in culture and in mouse skin xenografts following local subcutaneous injections (see below). However, it is not known if systemic treatment could be effective or beneficial for treating endogenous cancers as these could have acquired new mutations or changes that might alter their paradelopmental programs (see Ruiz i Altaba et al., 2004). Here we have chosen to use cyclopamine as the *in vivo* use of the more promising anti-GLI RNAi technology (Sanchez et al., 2004) is not yet satisfactory. The finding that ewes that ate the plant *Veratrum californicum*, and thus ingested cyclopamine and related compounds, gave birth to cyclopic offspring but appeared normal is encouraging (Keeler and Binns, 1968; Bryden et al., 1971). Similar results have been obtained with purified cyclopamine in other species (Keeler, 1970; Sim et al., 1983) after oral administration (Keeler and Baker, 1989). Consistent with the specific action of cyclopamine as a Hh pathway blocker, these cyclopic phenotypes mimic that of loss of Shh function in mice (Chiang et al., 1996) and humans (Belloni et al., 1996; Roessler et al., 1996).

Here we have used the *Ptc1*^{+/-}; *p53*^{-/-} mouse model of medulloblastoma (Goodrich et al., 1997; Wetmore et al., 2001) to test if systemic treatment by intraperitoneal injection of cyclodextrin-complexed cyclopamine (Van den Brink et al., 2001) is able to inhibit tumor growth once it has been initiated, to parallel the situation in human patients. We have also used a *p53* mutant background to include the loss of a tumor suppressor that is unrelated to the Hh-Gli pathway, thus allowing us to test if additional changes make the behavior of the cells forming the tumor derived from loss of *Ptc1* function independent of pathway activity. We demonstrate a clear reduction in tumor size as well as an inhibition of cell proliferation in the tumors *in situ*, demonstrating that systemic treatments with a Hh-Gli pathway blocker is beneficial as an anti-cancer strategy, showing that *ptc* mutant tumors having the additional loss of *p53* remain dependent of Hh pathway activity, and providing a solid base for therapeutic trials.

2. Results

2.1. Cyclopamine inhibits *Ptc1* expression and the proliferation of mouse medulloblastoma cells *in vitro*

Mice carrying a mutant allele of the *Ptc1* gene (*Ptc1*^{+/-}) often develop cerebellar tumors that closely resemble

human medulloblastomas (Goodrich et al., 1997; Hahn et al., 1998), the frequency of which increases to ~100% in the absence of *p53* (*Ptc1*^{+/-}; *p53*^{-/-}) (Wetmore et al., 2001). Consistently, we have observed that by 2 months of age, 95% of such mice, but not wild type or single *p53* mutant mice, display behavioral anomalies including circling, failure to regain an upright position when overturned and posterior paralysis. These animals have large cerebellar tumors that engulf the majority of the cerebellum, present an amorphous appearance with a smooth surface and often produce superficial local edemas, not present in control siblings (Fig. 1a,b), and die shortly afterwards due to medulloblastoma formation.

In order to test the potential of cyclopamine as an effective anti-tumor agent, we first confirmed its reported ability to inhibit tumor cell growth *in vitro* (Dahmane et al., 2001; Berman et al., 2002). Medulloblastomas from *Ptc1*^{+/-}; *p53*^{-/-} mice were dissected at ~7–8 weeks of age, dissociated and plated *in vitro* for primary culture. Dissociated cultures of normal cerebella from 2 month-old *p53*^{-/-} animals were prepared as control but these did not yield any cell growth (not shown) indicating that growth in the medulloblastoma cultures reflects the presence of tumor cells. Analyses of BrdU incorporation in the presence of 10 μ M cyclopamine demonstrated a reduction (between 50 and 80%; >1000 cells counted per sample; *n*=4 animals) in the proliferative capacity of tumor cells in the presence of cyclopamine as compared with carrier (ethanol)-only treated samples (Fig. 1c; each experiment was done in triplicate for each animal). In these mice, the *LacZ* gene disrupts the targeted *Ptc1* allele, reporting *Ptc1* transcription and thereby serving as a readout of pathway activity (Goodrich et al., 1997). All tumor cells were *LacZ*⁺ (e.g. Fig. 1e). The reduction in BrdU incorporation after cyclopamine treatment correlated with an obvious decrease (>60%) in the number and intensity of staining of β -galactosidase positive cells (Fig. 1e,f), indicating that cyclopamine is effective in inhibiting the Hh pathway. In two tumors tested, cyclopamine also induced apoptosis as measured by increased levels of activated Caspase 3 staining (Fig. 1d; 1000 cells counted per sample).

2.2. Intraperitoneal injections of cyclopamine reduce medulloblastoma tumor size *in vivo*

In order to test the anti-tumor capacity of cyclopamine *in vivo*, a 1 month-old *Ptc1*^{+/-}; *p53*^{-/-} mouse cohort was treated systemically with the drug or with the vehicle alone every other day. We have previously shown such *in vivo* treatment decreases Shh pathway activity in the brain as measured by a decrease in endogenous *Gli1* mRNA levels (Palma and Ruiz i Altaba, 2004; V. Palma et al., submitted). Injections were begun at 1 month of age, a time when the tumors are already detectable and the animals do not yet display any behavioral anomalies or signs of disease. In this way, we could test for possible beneficial effects on an

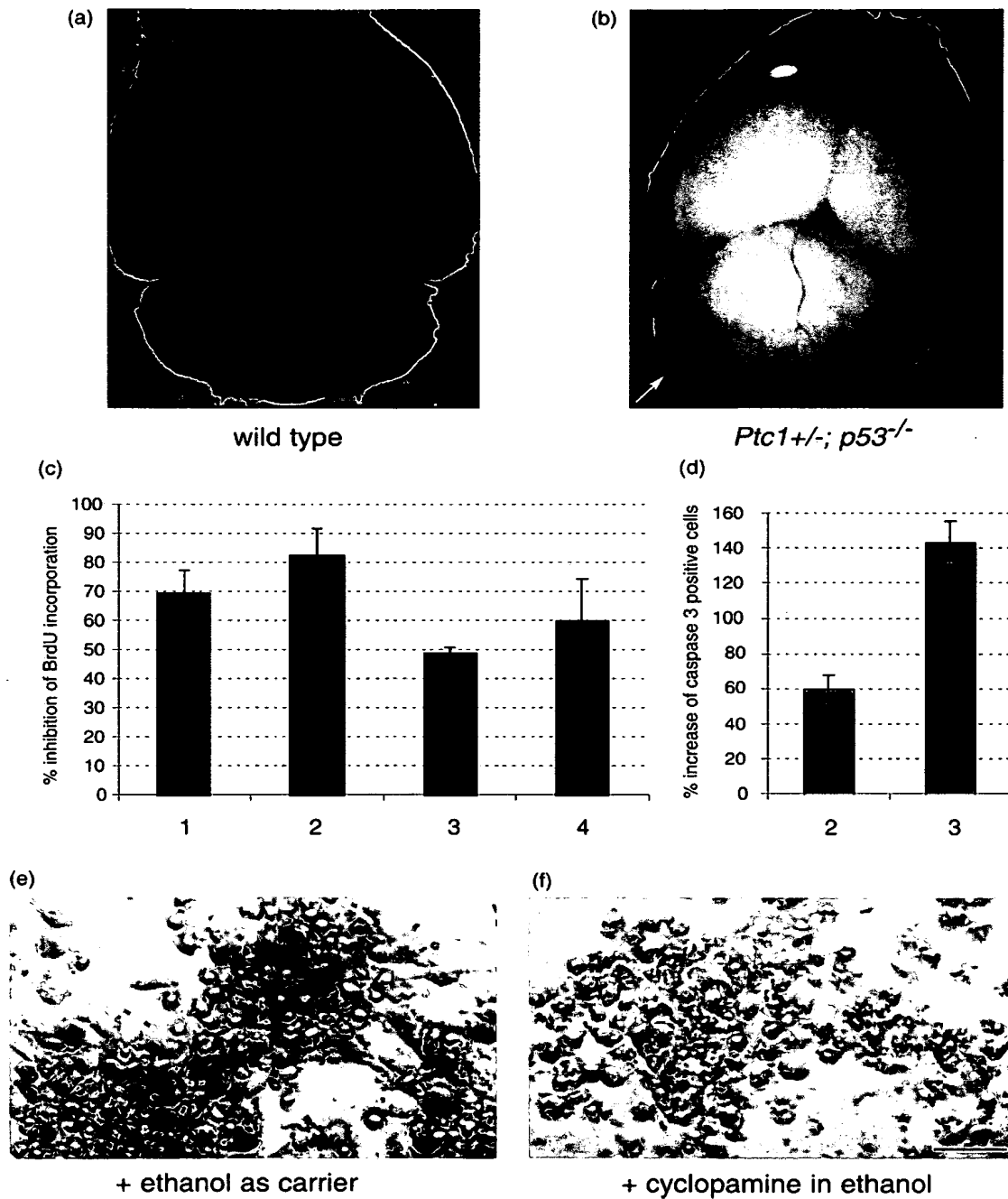


Fig. 1. Cyclopamine inhibits proliferation and survival of mouse medulloblastoma cells in vitro. (a,b) Dorsal view of a wild type (a) or a *Ptc1*^{+/-}; *p53*^{-/-} (b) mouse brains at 2 months of age. Note the large cerebellar tumor in the mutant (arrow, b). (c) Histograms of the inhibition of the proliferation of tumor cells in vitro by cyclopamine as assessed by BrdU incorporation, presented as percentage over carrier (ethanol)-treated samples. The cells were obtained from four different mouse medulloblastomas of *Ptc1*^{+/-}; *p53*^{-/-} mice. (d) Histogram of the increase in Caspase 3 immunolabeling, as a measure of apoptosis, in medulloblastoma cells obtained from two different mice, after treatment with cyclopamine for 48 h. The percentages denote increases over carrier-treated samples. (e,f) Example of x-gal staining revealing *Ptc1/LacZ* expression in primary mouse medulloblastoma cells treated with carrier only (e) or cyclopamine (f) for 48 h. Note that most tumor cells express the *Ptc1-LacZ* gene and that cyclopamine decreases its expression, indicating the effective inhibition of Hh pathway activity. Scale bar = 15 μ m for (e,f). All the changes are significant ($P < 0.05$, Student's *t*-test). All error bars represent SEM.

already initiated tumor in an otherwise intact brain, paralleling the human condition. After 1 month of treatment, at 2 months of age, we observed the typical medulloblastoma-associated abnormalities in carrier treated animals, including a decrease in overall activity, rough hair, decrease feeding, circling and the inability to gain an upright position when overturned, as well as signs of abnormal occipital prominences in the head. In contrast, cyclopamine-treated animals presented a near normal behavior at this time. Minor anomalies in gait and posture were seen in one animal resembling those of untreated cohorts animals with early medulloblastoma at ~1.3 months of age (not shown). This mouse had the largest tumor of the treated cohort (with an increase in ~23% of cerebellar volume over control; Fig. 3). This indicates that cyclopamine treatment is beneficial and that it is not toxic at the doses used even though it is predicted to affect other Hh-related events including, for example, the renewal of the gastric mucosa (Van den Brink et al., 2001). Animals were then sacrificed and their brains collected. The tumors of carrier-treated mice were apparent on gross examination (Fig. 2a), were prominent in sagittally bisected brains (Fig. 2d) and were similar to uninjected *Ptc1*^{+/−}; *p53*^{−/−} siblings of the same age (not shown). The brains of *Ptc1*^{+/−} control mice were normal or had minor signs of tumor (Fig. 2c). In contrast, cyclopamine-injected *Ptc1*^{+/−}; *p53*^{−/−} siblings had much smaller tumors and the foliation of the cerebellum was visible (Fig. 2b,e). The rest of the brain of cyclopamine- and carrier-injected animals, as well as the body appeared normal after a general pathological examination of all tissues and viscera (not shown). Similarly, wild type animals treated systemically with cyclopamine also did not show any gross pathological defects (Palma and Ruiz i Altaba, 2004). The present expense of cyclopamine and the length of time required to obtain *Ptc1*^{+/−}; *p53*^{−/−} cohorts made experiments with higher doses and longer treatments impractical. Histological analyses showed that the expression of β -galactosidase from the *LacZ* targeted *Ptc1* allele (Goodrich et al., 1997) was active in all tumors (Fig. 2f–h). In carrier treated animals the tumors invaded almost all the cerebellum (Fig. 2g), whereas in cyclopamine-treated mice the number of *lacZ* positive cells was severely reduced (Fig. 2h) indicating that the systemic injections of cyclopamine are able to inhibit the Hh pathway in the mice cerebella and that this inhibition is sufficient to curtail medulloblastoma growth.

To quantify the anti-tumor activity of cyclopamine in vivo we measured the cerebellar area of one sagittal section every 100 μ m for each animal and added the results for the whole cerebellum per animal to obtain a 'volume' value. *Ptc1*^{+/−}; *p53*^{−/−} mice showed an enhanced volume as compared to cerebella of sibling control animals due to the presence of medulloblastomas (Fig. 3a). No intracerebellar edemas or hemorrhages were detected upon sectioning (not shown). The size of the cerebellum of *Ptc1*^{+/−}; *p53*^{−/−} animals was as large as

175% that of normal siblings, whereas the size of the cerebella of cyclopamine-treated animals showed only a 10–20% increase, indicating an impairment of tumor growth by cyclopamine (Fig. 3a). The presence of remaining tumor cells in cyclopamine-treated brains that express β -gal (Fig. 2f) suggest that the doses used here inhibit tumor growth but do not shut down the Hh pathway completely as the normal endogenous expression of *Ptc1*–*LacZ* in the cerebellum is still present (Fig. 2).

2.3. Cyclopamine-treatment inhibits tumor cell proliferation in vivo

A decrease in tumor size could, in principle, result from a decrease in cell proliferation and/or an increase in cell death. Analyses of cell proliferation through phospho-Histone H3 immunolabeling, as a measure of cells in S phase, in cerebellar sections showed a reduction in cyclopamine versus carrier-treated samples after 1 month of treatment (Fig. 3b,c). In contrast, we were unable to detect a significant difference in the level of apoptosis between the two groups of animals as determined by Caspase 3 staining, (11.8 ± 2.2 positive cells per field (20 fields per brain) in HBC treated, versus 13.8 ± 4.8 in cyclopamine-treated mice ($P=0.7$).

3. Discussion

Solid cancers may be diseases of stem cell lineages in which paradevelopmental programs dictate the organization of tumoral tissues in attempts to recapitulate normal organogenesis (Ruiz i Altaba et al., 2004). In this case, there may be cancer stem cells that are responsible for the continued persistence of cancers and their recurrence after intervention, much as in the case of leukemias (discussed in Reya et al., 2001). In the brain, the possibility that brain tumors contain cancer-stem cells is supported by assays of self-renewal in vitro (Ignatova et al., 2002; Singh et al., 2003; Hemmati et al., 2003; P.S. and A.R.A., submitted). Moreover, we and others have shown that Hh-Gli signaling is required for normal precursor and stem cell behavior in the mouse brain (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Wallace, 1999; Dahmane et al., 2001; Lai et al., 2003; Machold et al., 2003; Palma and Ruiz i Altaba, 2004). Taken together, these findings suggest that Hh-Gli signaling blockade may be effective to combat cancer in vivo. Indeed, established cell lines, xenografts and primary culture assays strongly support this idea. However, in vivo data with endogenous tumors has been lacking. Here we demonstrate for the first time that anti-cancer therapy of a whole live animal with a Hh pathway inhibitor is effective and beneficial.

The tumor we have chosen to test derives from the loss of *Ptc1* function (perhaps through loss of heterozygosity or epigenetic silencing events (Berman et al., 2002), which

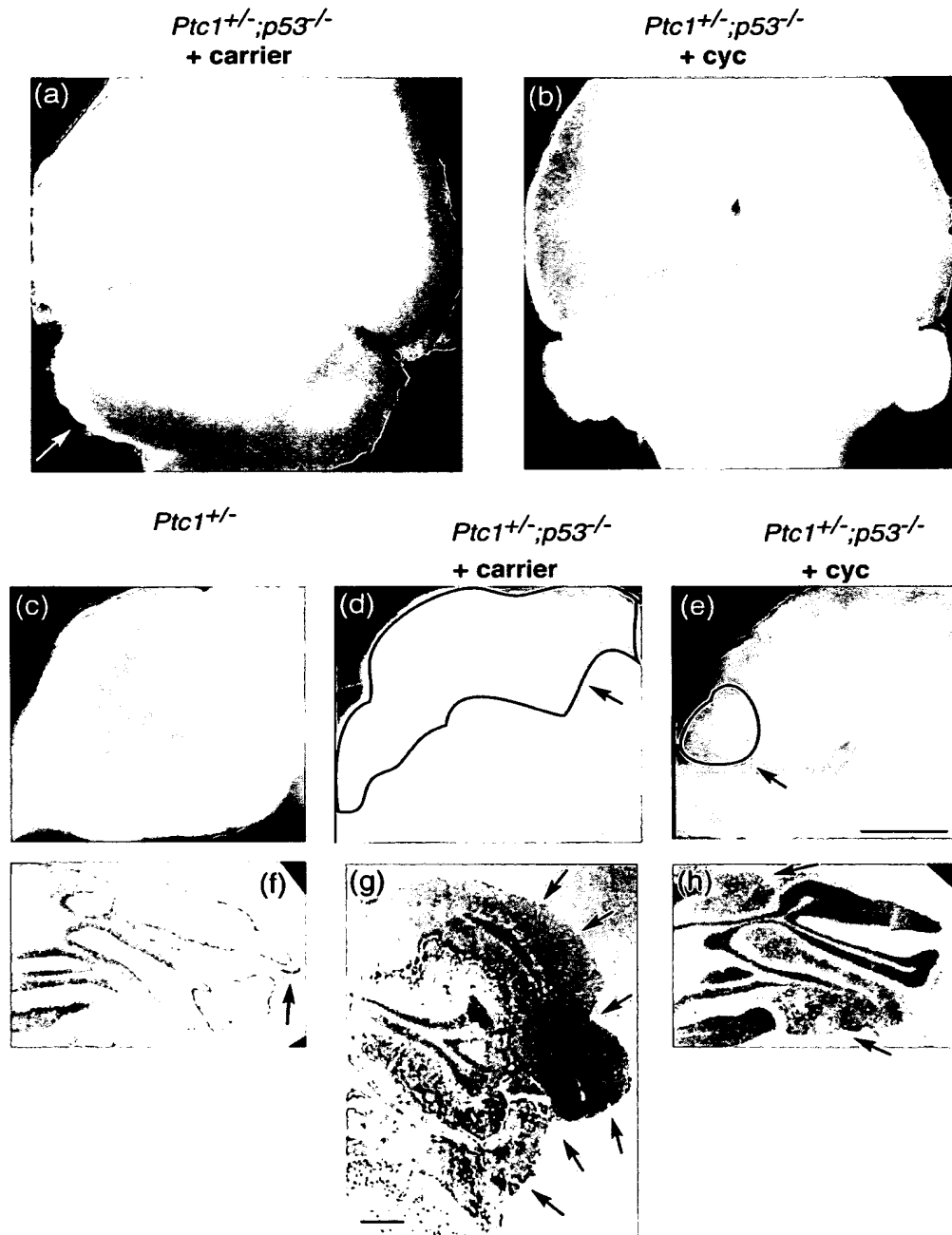


Fig. 2. Systemic cyclopamine treatment decreases tumor size in *Ptc1*^{+/-}; *p53*^{-/-} mice. (a,b) Example of the dorsal view of *Ptc1*^{+/-}; *p53*^{-/-} mouse brains treated for a month with cyclodextrin alone as carrier (a) or cyclodextrin-associated cyclopamine (b). The cerebellum of the cyclopamine-treated mouse appears almost normal (b) compared to the carrier treated in which a big tumor has deformed the normal foliation of the cerebellum (arrow, (a)). (c–e) Midline view of bisected cerebella of *Ptc1*^{+/-} (c) or *Ptc1*^{+/-}; *p53*^{-/-} mice treated with cyclodextrin alone (d) or cyclodextrin-associated cyclopamine (e). Foliation patterns are apparent in (c) and (e) only. Approximate tumor boundaries are denoted by a black line (arrows). (f–h) Cross-sections of cerebella shown in upper panels (c)–(e), respectively) after x-gal staining revealing *Ptc1/LacZ* expression. All tumors and normal granule cells express β -gal. Tumor areas are denoted by arrows. Scale bar = 150 μ m for (c)–(e), 40 μ m for (f)–(h).

translates into the activation of the Hh pathway at the level of the receptor complex (Goodrich et al., 1997). In addition, the complete homozygous loss of the p53 tumor suppressor gene accelerates tumor formation and the penetrance of

the tumorigenic phenotype to ~100% (Wetmore et al., 2001). Inhibition of such *Ptc1/p53* mutant tumors by blockade of the Hh pathway indicates that in tumors with at least two hits, as many others are likely to accumulate in

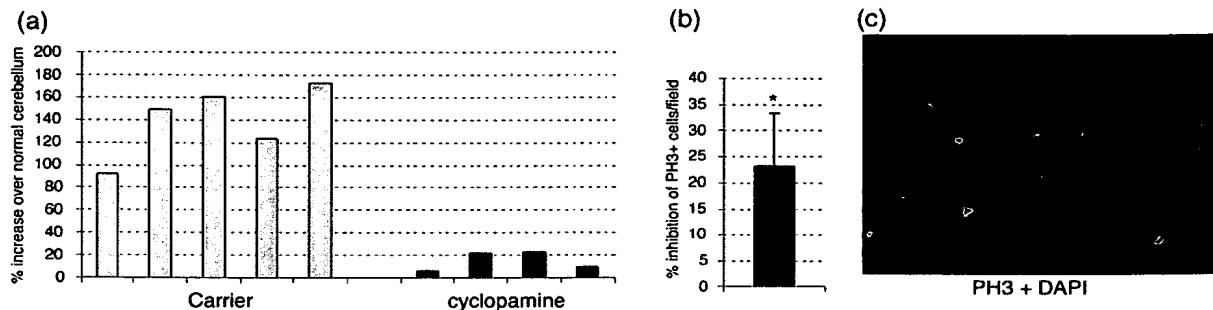


Fig. 3. Quantification of the effects of cyclopamine in vivo and effects on cell proliferation. (a) Quantification of cerebellar 'volume' in carrier and cyclopamine-injected *Pitd^{+/+};p53^{-/-}* mice as percentage of the normal weight of the cerebellum of wild type siblings. (b) Inhibition of proliferation as measured by phospho-Histone 3 expression in tumors by cyclopamine treatment in vivo, as those shown in (Fig. 2b,e,h), over carrier-treated controls (Fig. 2a,d,g). The increase is significant ($P < 0.05$). The relatively large error bar reflects differences in proliferation observed in different tumor areas. > 10 Sections were counted in two representative animals of each condition. (c) High-power photomicrograph of phospho-Histone H3 (PH3) immunolabeling of a section of medulloblastoma showing a number of positive nuclei (green). All nuclei are counterstained with the DNA dye DAPI (blue). Asterisks in the histograms in this and all figures denote significant changes ($P < 0.05$, Student's *t*-test).

the cells of tumor mass, cancer growth still requires sustained Hh signaling.

Sustained Hh-Gli signaling is involved in many different types of sporadic human tumors (Ruiz i Altaba et al., 2002b, 2004; Pasca di Magliano and Hebrok, 2003). These include those that can cause fulminant death such as gliomas (Dahmane et al., 2001; P.S. and A.R.A., submitted), medulloblastomas (Dahmane et al., 2001; Berman et al., 2002), pancreatic adenocarcinomas (Thayer et al., 2003), and prostate cancers, which are lethal in advanced stages and represent the most common tumor in men (Sanchez et al., 2004; Karhadkar et al., 2004), as well as the most common cancer type, sporadic basal cell carcinomas of the skin (Dahmane et al., 1997; Williams et al., 2003). Thus, the search for effective inhibitors of Hh signalling is a priority. Indeed, considerable effort is being devoted to the search and study of Hh pathway inhibitors that may be effective anti-cancer agents. Cyclopamine (Keeler and Binns, 1968; Keeler, 1978) is the first small molecule HH pathway blocker (Incardona et al., 1998; Cooper et al., 1998; Taipale et al., 2000) and it has proved efficient in inhibiting the proliferation of a variety of human cancer cells in vitro (e.g. Dahmane et al., 2001; Berman et al., 2002, 2003; Thayer et al., 2003; Watkins et al., 2003; Sanchez et al., 2004). It has also been used to inhibit the growth of mouse subcutaneous skin xenografts of medulloblastomas (Berman et al., 2002) and digestive tract and pancreatic human cancers (Thayer et al., 2003; Berman et al., 2003) after local subcutaneous injections. Our data provide a critical extension of these findings and show that systemic cyclopamine treatment inhibits the growth of endogenous Hh-dependent tumors. Thus, our results suggest that systemic or localized in vivo blockade of HH signaling will be also effective to combat many or all HH-related human cancers including those from the prostate, pancreas, lung, skin and brain.

The absence of significant increase in apoptosis in the cyclopamine-treated tumors in vivo that we have examined contrasts with the increase of activated Caspase 3 staining seen in vitro. This difference could be due to the action of different effective doses in the two scenarios. Indeed, apoptosis driven by Hh pathway blockade is often more evident in vitro after a prolonged period of treatment or at high doses (data not shown). It remains possible that the levels of inhibitors that reach the brain in our present protocol are not sufficient to induce apoptosis in tumor cells. This may be consistent with the detection of apoptosis by TUNEL assays in pancreatic tumor xenografts following subcutaneous cyclopamine delivery (Thayer et al., 2003) where the effective concentrations of the drug are likely to be much higher. Additional pharmacokinetic studies will be required to determine the amounts of cyclopamine, or of potential active byproducts, that reach the target cells in vivo, as well as the maximal tolerable doses. Notwithstanding, we show that systemic cyclopamine treatment affects the brain, with cyclopamine either crossing the blood/brain barrier or being effective in tumors that may have a broken barrier.

More generally, our data indicate that understanding normal embryonic development and organ function, including research into stem cells, is critical to understand diseases that may derive from mispatterning events such as cancer. Indeed, developmental biology suggest that cancers are cell group, tissue or organ diseases, in contrast to the view derived from cell biology which focused on cell intrinsic events such as the cell cycle. The present results thus urge the further exploration and definition of paradevelopmental programs that dictate tumor morphogenesis, as a parallel effort with the study of normal ontogeny, in order to have a full view of developmental potential. The data also beg for the initiation of clinical trials with Hh-Gli inhibitors for lethal cancers.

4. Experimental procedures

4.1. Animals

Ptc1^{+/-} mice were generated and maintained on a mixed C57B1/6/J background and crossed with mice carrying a targeted disruption in *p53* (Jackson Laboratories, Bar Harbor, ME) in the same background. Given the embryonic lethality of homozygote *Ptc1*^{-/-} mice and the small litter size, the generation of a 9 sibling *Ptc1*^{+/-}; *p53*^{-/-} cohort took ~15 months. At 2 months of age, control- and cyclopamine-treated animals were deeply anesthetized and brains fixed with 4% paraformaldehyde, removed and the presence of tumor confirmed by gross examination. Brains were then embedded in freezing medium (OCT), sectioned at 12 µm on a cryostat and processed for immunohistochemistry or Xgal staining. For cell culture, the animals were euthanized and the cerebellum dissected and its cells dissociated (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004).

4.2. Cell culture

Cells dissociated from dissected mouse medulloblastomas (taking only the core of the tumor mass) were plated at 40,000 cells/well onto polyornithine/laminin coated Lab-Tek chambers slides in Neurobasal Medium (see Palma and Ruiz i Altaba, 2004). Two days after dissociation, cells at 70% confluence were treated with 10 µM cyclopamine or carrier for 48 h. BrdU was added at 3 µM 16 h before fixation.

4.3. Cyclopamine treatment

Cyclopamine (Toronto Research Chemicals; ~760 \$/10 mg) and dissolved in ethanol for a stock at 10 mM for cell culture experiments or in a solution of 45% of 2-hydropropyl-β-cyclodextrin (HBC, Sigma) in PBS at 1 mg/ml by incubating 1 h at 65° for mouse injections (Van den Brink et al., 2001). Aliquots were stored at -20° until use. Animals were first injected at 1 month of age, when they are still asymptomatic, and on every other day afterwards, with 10 mg/kg of HBC-cyclopamine or the carrier alone, intraperitoneally (IP).

4.4. Immunohistochemistry

The following antibodies were used: monoclonal anti-BrdU (Becton-Dickinson), polyclonal anti-phospho-Histone3 (UPSTATE biotechnology), polyclonal anti-caspase3 activated (Cell Signaling), and fluorescein-conjugated secondary antibodies (Boehringer Mannheim). All probability values for the quantification of results were obtained using the Student's *t*-test.

4.5. X-gal staining

Cerebellar sections were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde, washed twice in phosphate buffer with deoxycolate and NP40, and incubated in the same buffer containing 1 mg/ml of X-Gal. Reactions were stopped after 3 h incubation at 37°.

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Cytotoxic effects induced by a combination of cyclopamine and gefitinib, the selective hedgehog and epidermal growth factor receptor signaling inhibitors, in prostate cancer cells

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Although the blockade of the hedgehog cascade by using cyclopamine has been reported to inhibit the growth of some cancer cell types, few studies on the mechanism by which this drug alone or in combination with other cytotoxic agents induces its cytotoxic effect have been reported. In our study, we evaluate, for the first time, the antiproliferative and cytotoxic effects induced by a combination of selective SMO inhibitor, cyclopamine and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, gefitinib on metastatic prostate cancer (PC) cells. The results revealed that cyclopamine, alone or at a lower concentration in combination with gefitinib, inhibited the growth of sonic hedgehog- (SHH), epidermal growth factor- (EGF) and serum-stimulated androgen-sensitive LNCaP-C33 and LNCaP-LN3 and androgen-independent LNCaP-C81, DU145 and PC3 cells. The antiproliferative effect of cyclopamine and gefitinib, alone or in combination, was mediated *via* a blockade of the PC3 cells in the G1 phase of the cell cycle. Importantly, the combined cyclopamine and gefitinib also caused a higher rate of apoptotic death of PC cells compared to single agents. The cytotoxic effect induced by these drugs in PC3 cells appears to be mediated at least, in part, *via* the mitochondrial pathway through the depolarization of the mitochondrial membrane and the release of cytochrome *c* and reactive oxygen species into the cytosol. This was also accompanied by the activation of caspase cascades, PARP cleavage and DNA fragmentation. Additionally, the combined cyclopamine and gefitinib were more effective at suppressing the invasiveness of PC3 cells through matrigel *in vitro* as the drugs alone. These findings indicate that the simultaneous blockade of SHH–GLI-1 and EGF–EGFR signaling, which results in the growth arrest and massive rate of apoptotic cell death, represents a promising strategy for a more effective treatment of metastatic PC forms.

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Key words: prostate cancer; hedgehog signaling; cyclopamine; EGFR; growth inhibition; apoptotic death

The mammalian hedgehog (HH) family of genes encodes several secreted proteins, including Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH) proteins, which are expressed in a tissue-specific manner. These HH products are important signaling elements for normal embryonic development and neoangiogenesis.^{1–4} In particular, the SHH protein is expressed in an embryo and participates in regulating cell proliferation, differentiation and tissue patterning of many organs including the prostate gland, pancreas, lung, biliary tract and brain.^{1,4–9} The SHH ligand mediates its biological effects through the multi-component receptor complex constituted from a transmembrane protein, PATCHED-1 (PTCH-1). PTCH-1 binds SHH with high affinity and Smoothened (SMO), a second signaling transmembrane G-protein coupled receptor.^{10–12} In the absence of SHH, PTCH-1 represses SMO activity, while the binding of SHH to PTCH-1 releases the basal repression of SMO by PTCH-1. This ultimately leads to the activation of the GLI-1 transcription factor, which induces the expression of numerous target genes that regulate proliferation, differentiation and extracellular matrix interactions.^{10,11,13,14}

Although the HH developmental pathway appears to be generally downregulated in adults, recent evidence indicated that the increased expression and deregulated activation of HH signaling

elements could be involved in regeneration processes and the development of distinct metastatic cancers including carcinomas of the prostate.^{1,5,11–23} In this matter, the decreased expression of the fused Su(Fu) protein, which interacts with GLI and thereby inhibits GLI-mediated gene transactivation, appears notably to be associated with upregulating HH signaling and enhanced tumor angiogenesis.^{24–26} In particular, the *hSu(fu)* gene is localized at chromosomal region 10q24–25, which is inactivated through mutations in several cancer types including certain prostate cancer (PC) forms.^{24,26} Additionally, the epidermal growth factor receptor (EGFR) and its ligands, epidermal growth factor (EGF) and transforming growth factor- α (TGF- α), which assume an important role for normal prostate development,^{27–30} are also overexpressed during the progression of benign, localized and metastatic forms of PC.^{31–34} The upregulated activation of EGFR signaling appears notably to contribute to the sustained cell proliferation, survival and invasion of PC epithelial cells.^{27,29,31–33,35,36} It has also been reported that the blockade of either HH or EGF–EGFR signaling, by using a selective SMO or EGFR inhibitor, such as cyclopamine and gefitinib (also known as Iressa or ZD1839), respectively, leads to the growth inhibition and apoptotic death of many cancer cell types, including prostate tumor epithelial cells *in vivo* and *in vitro*.^{17,21,33,35,37–41} In addition, our previous work has also revealed that the combined use of low concentrations of EGFR inhibitor with the protein kinase A inhibitor (Rp-cAMPs) or sodium nitroprusside resulted in the growth arrest and a more substantial rate of PC cell death as compared to the drugs alone.^{39,40}

The present investigation was undertaken to determine whether the simultaneous blockade of SHH–GLI-1 and EGF–EGFR pathways might effectively counteract the PC cell growth and survival induced by different growth factors than single agents. Therefore, the antiproliferative and cytotoxic effects induced by cyclopamine, alone or in combination with selective EGFR inhibitor gefitinib, were estimated on the androgen-responsive LNCaP-C33 and LNCaP-LN3 and androgen-independent LNCaP-C81, DU145 and PC3 PC lines in serum-free medium and stimulated by SHHNP, EGF and serum. Moreover, the molecular mechanisms involved in the cytotoxic effects of cyclopamine and gefitinib were studied in metastatic PC3 cells.

Material and methods

Materials

The human PZ-HPV-7 nonmalignant prostate epithelial (PrE) cells and LNCaP-FGC (LNCaP), DU145 and PC3 PC cell lines

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were originally purchased from American Type Culture Collection (ATCC, Manassas, VA), while androgen-responsive LNCaP-C33 and androgen-independent LNCaP-C81 cells were developed at UNMC.⁴² The LNCaP cells that had a passage number less than 37 were designated as C-33, and those with a number over 80 as C-81 cells.⁴² The metastatic LNCaP-LN3 cells (obtained from Dr. Fidler, M.D. Anderson Cancer Center, TX) were generated by the repeated lymph node metastasis of the LNCaP cells grown orthotopically in the prostate gland of athymic mice. The LNCaP-LN3 cells exhibited low local aggressiveness, but significantly increased metastatic potential, less sensitive to androgen *in vitro* and *in vivo*, secrete high levels of PSA, and have the ability to metastasize into the liver even after intrasplenic implantation.⁴³ The PC cells were maintained routinely in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS), 26 mM NaH₂CO₃ pH 7.4, 1% L-glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) in a 37°C incubator supplied with 5% CO₂. PZ-HPV-7 cells were cultured in keratinocyte-SFM medium supplemented with L-glutamine, bovine pituitary extract and EGF, according to the manufacturer's instructions. Cyclopamine was obtained from Toronto Research Chemicals, Inc. (Brisbane, Canada). Fumonisin B1, catalase, dihydroethidine (HEt), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 3',3'-diethylxycarbocyanine iodide (DiOC₆(3)), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and EGF were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The N-terminal peptide of recombinant human Sonic hedgehog (SHHNP) was obtained from R&D Systems (Minneapolis, MN) and the broad caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), from Calbiochem (San Diego, CA). The selective EGFR tyrosine kinase inhibitor, gefitinib, was synthesized according to a modification of a described procedure.⁴⁴ RPMI 1640 medium and all other cell culture materials were from Life Technologies (Invitrogen Corp., Carlsbad, CA). The amounts of proteins were estimated by using a DC Protein Assay kit from Bio-Rad Laboratories (Hercules, CA). The ELISA kit for measurements of cytosolic cytochrome *c* was purchased from Zymed Laboratories, BD BioCoat™ matrigel™ invasion chamber and control insert chamber from Becton Dickinson Labware (San Jose, CA) and Diff-Quick™ stain set from Dade Behring Corp. (Deerfield, IL). Human polyclonal antibody directed against 110 kDa human poly (ADP-ribose) polymerase, PARP(197-214) protein, or the cleaved 29 kDa PARP fragment was purchased from Calbiochem. All other reagents were purchased from Sigma-Aldrich.

Reverse transcriptase-polymerase chain reaction analyses

PC cells were maintained in medium containing 10% FBS for 48 hr, and the expression levels of HH signaling elements, as well as EGFR, were estimated by reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. Moreover, PC3 cells were also starved for 24 hr and then treated with 40 nM SHHNP in the absence or presence of 10 µM cyclopamine for an additional 48 hr. After incubation, the cells were collected by centrifugation, and the total cellular mRNA was extracted from cultured cell pellets using the RNeasy kit (Qiagen), according to the manufacturer's instructions. RT-PCR was performed with the SuperScript™ II reverse transcriptase and Taq DNA Polymerase (Invitrogen) on 2 µg of total RNA. The reaction medium contained 2 µl of each primer. Equivalent amounts of primers (5 nmol/µl) were added to a 40.5 µl master mix of PCR reagents. After the denaturation of the aliquots at 95°C for 10 min, RT-PCR was performed as follows: reverse transcription at 48°C for 1 hr, denaturation at 95°C for 45 sec and annealing/extension at 59°C for 1 min (repeated 35 cycles) (SHH, PTCH-1, SMO, GLI-1), 60°C for 45 sec (repeated 30 cycles) (EGFR) or 60°C for 45 sec (repeated 25 cycles) (β-actin). The samples were analyzed by electrophoresis on a 1.5% agarose gel staining with ethidium bromide. The primer sequences used to estimate the mRNA expression levels of human signaling products were as follows: 5'-3' for 381 bp SHH (forward: GATGGCCACCACTC A GAGGAG; reverse: CGTC-

TCGATCACAGTAGAAGAC), 265 bp PTCH-1 (forward: TTCTCACAA CCCTCGGAACCCA; reverse: CTGCAGCTCAATGAC-TTCCACCTTC), 263 bp SMO (forward: ATCTCCACAGGAGAG-ACTGGTTCGG; reverse: AAAGTGGGCCTTGGGAACATG), 351 bp GLI-1 (forward: TACTCAGCCTCGAAACCT; reverse: GTCTGCTTTCCTCCCTGATG), 345 bp EGFR (forward: ATG-TCCGGGAACACAAAGAC; reverse: TTCCGTCATATGGCT-TGGAT) and 541 bp β-actin (forward: GTTGTATCCAGGCT-GTGC; reverse: GCATCCTGT CCGCAATGC).

Cell culture and growth assays

All PC cells were maintained in RPMI 1640 culture medium containing 10% FBS as mentioned earlier. For growth assays, the cells were seeded on 96-well plates at a density of $\sim 3-5 \times 10^4$ cells/well in a total volume of 200 µl. After 3 days, the cell culture medium was changed to serum-free medium, and the cells were starved for 24 hr for the synchronization. The cell growth assays were then performed in the serum-free medium or medium containing 40 nM SHHNP, 10 ng/ml EGF or 10% FBS. Different cyclopamine concentrations, alone or in combination with 0.1 µM gefitinib, were also added to the culture medium. The concentration of 0.1 µM gefitinib used in these experiments was selected on the basis of our previously published results, indicating that this concentration is inducing a significant inhibitory effect on the proliferation of PC cells.⁴⁰ After an additional 48 hr, the rate of cell growth was estimated by MTT colorimetric test.⁴⁵

Flow cytometric analyses

All PC cells were grown at a density of about 5×10^5 cells on 25 cm² dishes as described earlier, synchronized during 24 hr in serum-free medium and treated with different concentrations of cyclopamine, alone or in combination with 0.1 µM gefitinib, in the absence or presence of Z-VAD-fmk, catalase or fumonisin B1 for 4 days. In all experiments, the cells were kept at subconfluent to avoid contact inhibition. Moreover, in order to determine the influence of cyclopamine on the cellular cycle progression of SHHNP- and EGF-stimulated PC3 cells, the cytometric analyses by fluorescence-activated cell sorting (FACS) were performed 24 hr after the addition of 2 or 5 µM cyclopamine and 0.1 µM gefitinib, alone or in combination. The DNA content analysis of each sample was performed by FACS™ essentially as previously described.^{39,40} In addition, the percentage of cell death was also estimated by the amount of trypan blue-stained cells.

Estimation of mitochondrial transmembrane potential

To determine whether the apoptotic effect induced by cyclopamine, alone or in combination with gefitinib, in PC3 cells is mediated *via* a mitochondrial pathway, the mitochondrial membrane potential (MMP) was estimated by using the cationic, lipophilic and fluorescent dye, DiOC₆(3), which specifically accumulates within the mitochondrial compartment in a MMP-dependent manner.⁴⁶ Briefly, PC3 cells were untreated (control) or treated with 5 or 10 µM cyclopamine and 1 µM gefitinib, alone or in combination, in the absence and presence of irreversible and broad caspase cascade inhibitor, Z-VAD-fmk, for 4 days, and the accumulation of DiOC₆(3) within the mitochondria was measured by a FACScan flow cytometer, as previously described.^{39,40}

Estimation of cytosolic cytochrome *c* release by ELISA tests

After 4 days of PC3 cell growth on 25 cm² dishes in the absence or presence of different tested agents, which was performed under the same conditions as described above, the floating and adherent PC3 cells were collected by centrifugation, rinsed twice with phosphate buffered saline (PBS) and centrifuged. Then, the amounts of cytochrome *c* present in the cytosolic extracts of each sample were estimated following the method described in the ELISA kit from Zymed Laboratories with a human anticytochrome *c* antibody.

Estimation of intracellular reactive oxygen species

PC3 cells were untreated (control) or treated with 5 μ M cyclopamine and 1 μ M gefitinib, alone or in combination, for 4 days as mentioned earlier, and the floating and adherent cells detached by trypsination were collected and washed in PBS. The pellets corresponding to $\sim 1 \times 10^6$ cells were resuspended in 1 ml PBS, containing 10 μ M H₂O₂ or 5 μ M DCFH-DA dye. These probes allow the estimation of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) production, respectively, by measuring the increase in fluorescence associated with the oxidation of dihydroethidium (H₂Et) to ethidium (Et) and DCFH-DA to 2',7'-dichlorofluorescein (DCF).^{46,47} After the incubation of cells at 37°C for 20 min, the intracellular $O_2^{\cdot -}$ and H_2O_2 levels were monitored by cytofluorometric analyses changes in the Et or DCF fluorescence intensity emitted in red and green, at a wavelength of 630 and 525 nm, respectively.

Western blot

The PC3 cells were grown at a density of about 5×10^5 cells on 25 cm² dishes, starved during 24 hr in serum-free medium and treated with different concentrations of cyclopamine, alone or in combination with gefitinib, for 4 days. Total PC3 lysate samples were prepared by lysing cells in 50 mM Tris-HCl pH 7.4 buffer containing 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). The lysates were centrifuged at 10,000g for 30 min to remove insoluble material. The samples corresponding to 20 μ g cell lysates were diluted in reductor Laemmli sample buffer and resolved on a 10% polyacrylamide gel and transferred to an immobilon-P transfer membrane. After blocking the membrane with 5% nonfat dry milk, Western blots were incubated overnight with human polyclonal antibody for intact and cleaved PARP. At the end of incubation, the blot was washed in 0.05% Tween-20 in PBS and incubated with human antirabbit IgG for 1 hr. Antibody-antigen complexes were visualized using enhanced chemiluminescence technique with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ).

Electrophoretic analysis of DNA fragmentation

The qualitative analysis of DNA fragmentation associated with the apoptotic death of PC3 cells induced by cyclopamine, alone or in combination with gefitinib, was also made by the electrophoretic method essentially as previously described.^{39,40} Briefly, PC3 cells were grown during 4 days without agents (control) or treated with 5 or 10 μ M cyclopamine and 1 μ M gefitinib, alone or in combination, in the absence or presence of broad caspase inhibitor, 50 μ M Z-VAD-fmk. The adherent and floating PC3 cells were collected by centrifugation, and DNA was extracted from each sam-

ple and analyzed on a 1.5% agarose gel electrophoresis. The gel was visualized on UV light after staining with ethidium bromide, and the DNA patterns were photographed.

In vitro invasion assays

The invasive potential of PC3 cells was estimated by their ability to penetrate a Matrigel-invasion chamber with a Falcon cell culture insert 8 μ m pore size PET membrane, with a thin layer of matrigel matrix acting as a basement membrane *in vivo*, and allowing an estimate of the metastatic potential of tumor cells *in vitro*.⁴⁸ PC3 cells were untreated (control) or pretreated with 2 or 5 μ M cyclopamine and 0.1 μ M gefitinib, alone or in combination for 24 hr, and during cell invasion assay for an additional 24 hr. For each experiment, 2.5×10^5 PC3 cells/well in a total volume of 2 ml serum-free medium without drugs (control) or containing the drugs were loaded into the top of the BD BioCoat Matrigel cell invasion chamber or control insert chamber without matrigel, according to the manufacturer's instructions. At the end of incubation, the invasive cells reaching the lower chamber were stained with a Diff-Quick stain set and counted using a hemocytometer by phase-contrast microscopy. The percentage of cell invasion was calculated by dividing the mean of cell numbers invading through the matrigel insert membrane by the mean of cell numbers migrating through the control insert chamber. Student's *t*-test was used to compare the results, with *p* values < 0.05 indicating statistically significant differences.

Results

Expression levels of HH signaling elements and EGFR in different PC cell lines

The expression levels of HH and EGFR signaling components were examined by RT-PCR analyses in PC cell lines maintained in 10% FBS. As shown in Figure 1a, the significant levels of SHH ligand, PTCH-1 receptor, SMO and GLI-1 as well as EGFR were detected in LNCaP-C81, LNCaP-LN3, DU145 and PC3 cells while the endogenous expression of these signaling elements was weaker in androgen-sensitive LNCaP-C33, and very low to undetectable for immortalized PZ-HPV-7 cells. Moreover, the treatment of SHHNP-stimulated PC3 cells with cyclopamine, a SMO inhibitor, also induced a decrease of the PTCH-1 and GLI-1 expression levels as compared to those observed for SHHNP-stimulated PC3 cells untreated by cyclopamine (Fig. 1b). Additionally, the EGFR level was also reduced in SHHNP-stimulated PC3 cells in the presence of cyclopamine (Fig. 1b).

Antiproliferative effect induced by cyclopamine and gefitinib in the different PC cells

To estimate the implication of HH and EGFR signaling in the proliferation of metastatic PC cells, the growth-inhibitory effects of cyclopamine and gefitinib, alone or in combination, were eval-

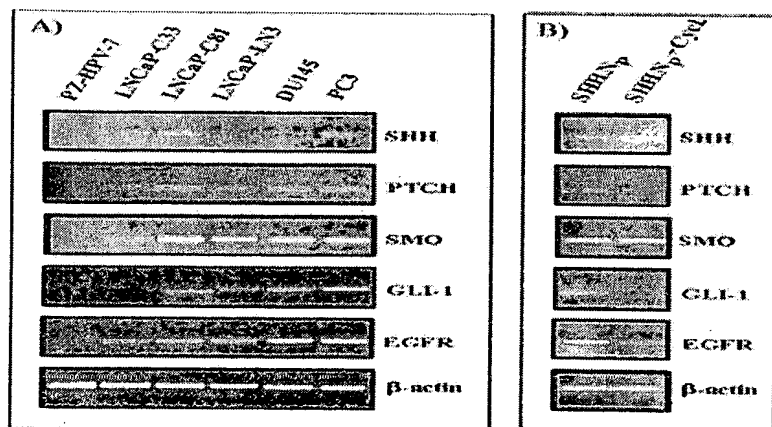


FIGURE 1 – RT-PCR analyses of expression levels of Hedgehog and EGFR signaling components in PC cell lines. The cell lines were maintained for 48 hr in (a) 10% FBS or (b) 40 nM SHHNP in the absence and presence of 10 μ M cyclopamine for PC3 cells. Then, mRNA expression levels of HH signaling components and EGFR in nonmalignant PZ-HPV-7 cells and LNCaP-C33, LNCaP-C81, LNCaP-LN3, DU145 and PC3 PC cell lines were estimated by the RT-PCR analyses as described in Material and methods. The representative results obtained from 3 separate experiments are shown.

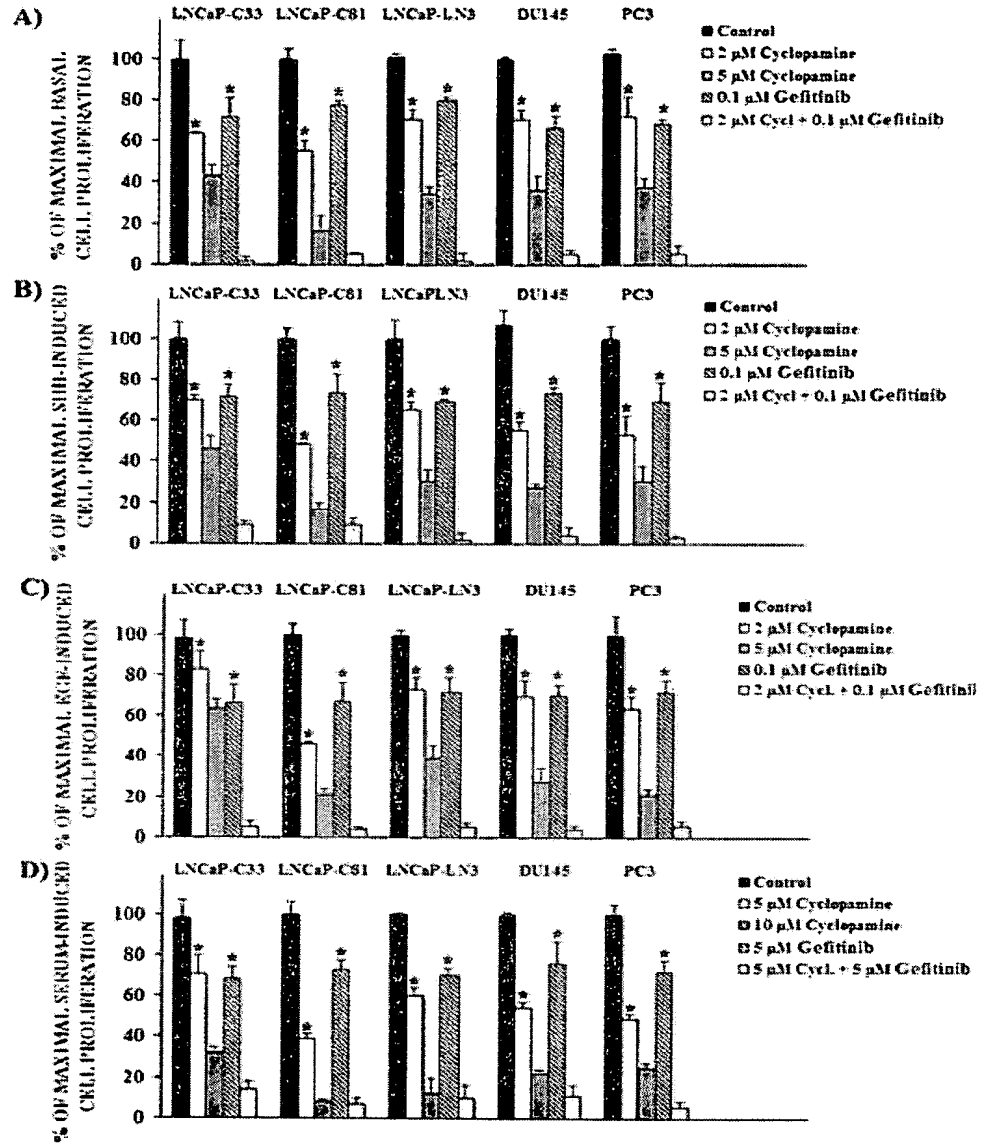


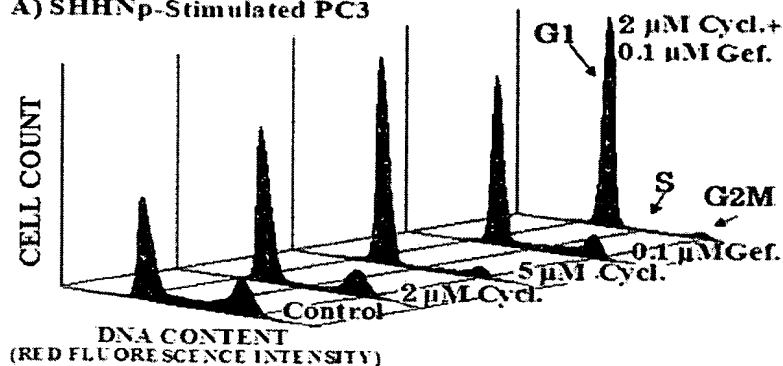
FIGURE 2 – Antiproliferative effect of mixed cyclopamine and gefitinib on the proliferation of (a) serum-free medium- (basal), (b) SHHNP-, (c) EGF- and (d) 10% serum-stimulated PC cells. The PC cells were treated with the indicated concentrations of cyclopamine (Cycl.) and gefitinib, alone or in combination, for 2 days. Estimation of the PC cell growth was performed after 2 days of the drug treatment by MTT colorimetric tests. The effects of tested agents are expressed as the percentage of inhibition of serum-free medium- (basal), SHHNP-, EGF- and 10% serum-induced PC cell proliferation as compared to the growth of untreated control PC cells. Data are means $\pm$ SE of 3–4 different experiments performed in triplicate. * $p < 0.005$, indicates significant difference between the effects obtained for the cyclopamine and gefitinib alone, as compared to the combined agents.

uated on the serum-free medium- (basal), SHHNP-, EGF- and serum-stimulated PC cells. As shown in Figure 2, the cyclopamine inhibited the growth of androgen-responsive LNCaP-C33 and LNCaP-LN3 and androgen-independent LNCaP-C81, DU145 and PC3 in a concentration-dependent manner. Moreover, the combination of 2 μ M cyclopamine plus 0.1 μ M gefitinib induced a supra-additive growth inhibitory effect on the serum-free medium- (basal), SHHNP- and EGF-stimulated PC cells. As shown in Figure 3, the inhibition of SHHNP- and EGF-stimulated PC3 cell growth induced by 2 and 5 μ M cyclopamine for 24 hr was also accompanied by increasing the cell population in the G1 phase of cell cycle, concomitant with a reduction of PC3 cell populations in the S and G2M phases. Of particular interest, 2 μ M cyclopamine plus 0.1 μ M gefitinib caused an arrest of the growth *via* a blockade in the G1 phase of the cell cycle (Fig. 3). In addition, 5 or 10 μ M cyclopamine also inhibited the serum-stimulated PC cell proliferation (Fig. 2d). Moreover, 5 μ M cyclopamine plus 5 μ M gefitinib induced a greater inhibitory effect as compared to individual agents on serum-stimulated PC cells.

Apoptotic effect induced by cyclopamine and gefitinib in the different PC cells

To estimate the percentage of apoptotic cell death induced by cyclopamine, alone or in combination with gefitinib, the flow cytometric analyses were made, and the apoptotic cell number in sub-G1 phase was quantified. As shown in Figure 4, the treatment of all PC cells for 4 days with cyclopamine caused, in a concentration-dependent manner, an increase in apoptotic cell population as compared to untreated cells (control). In particular, concentrations up to 10 μ M cyclopamine were necessary for the induction of apoptosis in the androgen-responsive LNCaP-C33, while 5–10 μ M cyclopamine induced a significant rate of apoptotic cell death in the more metastatic LNCaP-LN3 and androgen-independent LNCaP-C81, DU145 and PC3. Importantly, 5 μ M cyclopamine plus 1 μ M gefitinib was more cytotoxic to all tested PC cells than agents alone, and this combination caused the death of the majority of cells. Additional evidence that cyclopamine and gefitinib induces apoptosis in PC3 cells is also indicated by the increase in cleaved PARP and DNA fragmentation observed in the

A) SHHNp-Stimulated PC3



B) EGF-Stimulated PC3

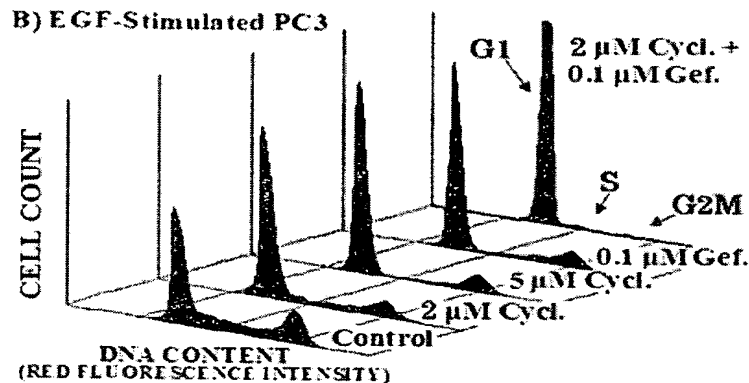


FIGURE 3 – FACS analyses of the inhibitory effect induced by mixed cyclopamine and gefitinib on (a) SHHNp- and (b) EGF-stimulated PC3 cells. The inhibitory effect induced by drugs on the progression of SHHNp- and EGF-stimulated PC3 cells in the cellular cycle was investigated by flow cytometric analysis. The stimulated PC3 were untreated or treated with 2–5 μ M cyclopamine (Cycl.) or 0.1 μ M gefitinib (Gef.), alone or in combination, for 24 hr. At the end of incubation time, the lysates were prepared as described in Materials and methods, and the cell cycle distributions were assessed by FACS analyses. The representative results obtained from 3 separate experiments are shown.

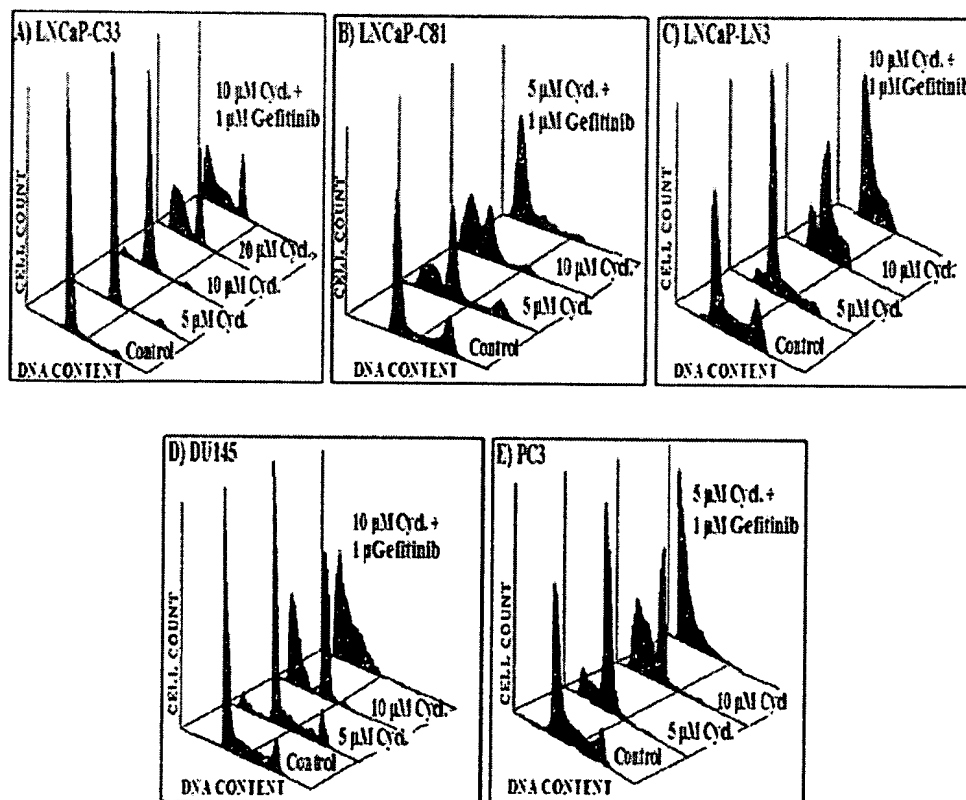


FIGURE 4 – FACS analyses of the apoptotic effect induced by cyclopamine and gefitinib in (a) LNCaP-C33, (b) LNCaP-C81, (c) LNCaP-LN3, (d) DU145 and (e) PC3 cell lines. The PC cells were untreated (control) or treated with the indicated concentrations of cyclopamine (Cycl.) alone or plus 1 μ M gefitinib for 4 days. Then, the cells nuclei were stained with propidium iodide, and the DNA content was analyzed by flow cytometry.

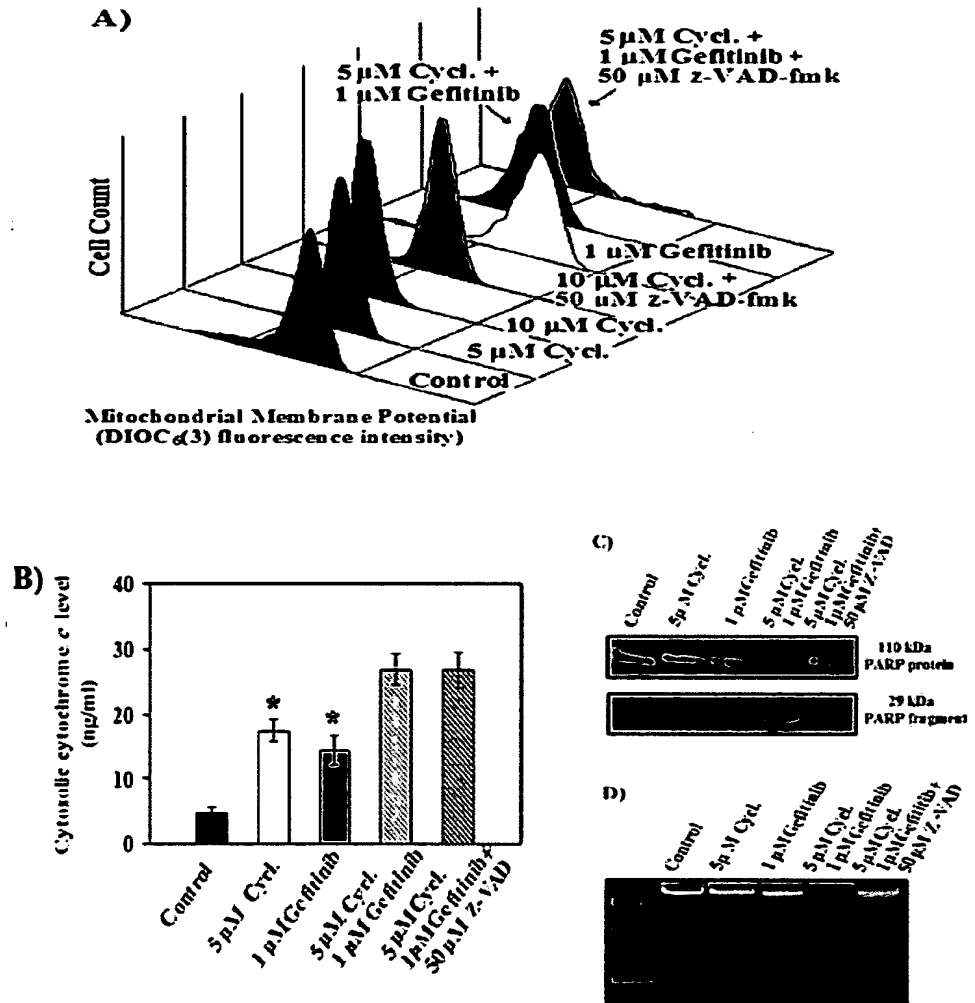


FIGURE 5 – Stimulatory effect induced by cyclopamine and gefitinib on (a) MMP, (b) cytosolic cytochrome *c* releasing, (c) PARP cleavage and (d) cellular DNA fragmentation. The PC3 cells were untreated (control) or treated with 5–10 μM cyclopamine (Cycl.) or 1 μM gefitinib, alone or in combination, in the absence and presence of 50 μM Z-VAD-fmk for 4 days. After the treatments, the cells were prepared by staining with 40 nM DIOC₆(3) for analyses of mitochondrial potential by flow cytometry. Moreover, the amounts of cytochrome *c* released into cytosol were estimated by ELISA and DNA laddering analyzed by agarose gel electrophoresis, as described in Materials and methods. The representative data (a, c and d) and results (b) obtained from 2 to 3 separate experiments are shown. **p* < 0.05, indicates significant difference between the effects observed for the cyclopamine and gefitinib alone, as compared to the combined drugs at same concentrations.

presence of these drugs as compared to untreated cells (Fig. 5). Comparable results were also obtained from the trypan blue exclusion analyses of PC3 cell viability after 4 days (Table I).

Estimation of the role of the mitochondria and caspase cascades in the apoptotic effect induced by cyclopamine and gefitinib in PC3 cells

To assess whether the mechanisms whereby cyclopamine, alone or in combination with gefitinib, induces cell death involving the mitochondria and caspase pathways, the effect of the drug treatment on the mitochondrial transmembrane potential (MMP), cytochrome *c*, reactive oxygen species (ROS) levels and DNA fragmentation was estimated in PC3 cells. The results indicated that the continuous treatment of PC3 cells for 4 days with 5 μM cyclopamine and 1 μM gefitinib alone caused only a slight decrease of MMP, as indicated by the weak shoulder of peak as compared to the stained PC3 cells untreated (control) (Fig. 5a). The treatment of PC3 with 10 μM cyclopamine was, however, accompanied with a marked decrease of MMP. Moreover, the addition of 5 μM cyclopamine plus 1 μM gefitinib induces a higher mitochondrial membrane depolarizing effect and a higher amount of cytochrome *c* released into the cytosol as compared to the treatment of PC3 cells with individual drugs (Figs. 5a and b). In addition, the combination of cyclopamine and gefitinib induced

TABLE I – EFFECT OF VARIOUS AGENTS ON CELL DEATH INDUCED BY CYCLOPAMINE AND GEFITINIB IN PC3 CELLS

Tested agent	Cell death ¹ (%)	
	FACS	Trypan blue exclusion
PC3 (untreated)	5 ± 2	3 ± 1
10 μM Cyclopamine	44 ± 2	48 ± 4
+ 50 μM Z-VAD-fmk	7 ± 1	6 ± 2
+ Catalase (500 Unit/ml)	33 ± 2	34 ± 3
+ 10 μM Fumonisin B1	37 ± 5	50 ± 5
5 μM Cyclopamine	29 ± 1	31 ± 2
1 μM Gefitinib	25 ± 3	24 ± 4
5 μM Cyclopamine plus 1 μM Gefitinib	83 ± 10	89 ± 3
+ 50 μM Z-VAD-fmk	14 ± 2	10 ± 4
+ Catalase (500 Unit/ml)	55 ± 1	62 ± 6
+ 10 μM Fumonisin B1	84 ± 5	91 ± 1

¹Data represents the percentage of PC3 cell death by apoptosis and/or necrosis observed for different tested agents at day 4 as estimated by the cell counts in sub-G1 phase following FACS analyses and trypan blue method. Data are mean ± SE of 2–4 different experiments.

a significant increase of the intracellular O₂^{•−} and H₂O₂ levels estimated for PC3 cells as compared to individual agents (*p* < 0.005) (Fig. 6). In particular, the PC3 cell population was enhanced about 6.9-fold in Et red fluorescence and 18.4-fold in DCF green fluores-

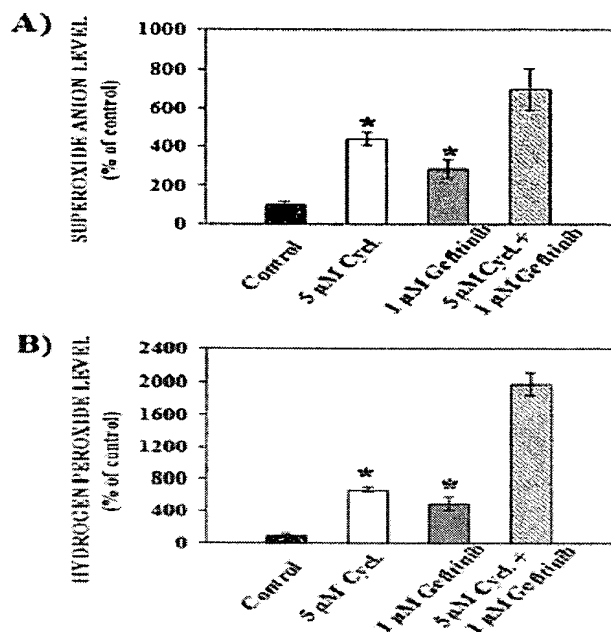


FIGURE 6 – Stimulatory effect of cyclopamine and gefitinib on production of (a) intracellular $O_2^{\cdot -}$ and (b) H_2O_2 levels in PC3 cells. The PC3 cells were untreated (control) or treated with 5 μ M cyclopamine (Cycl.) or 1 μ M gefitinib alone or in combination for 4 days. At the end of treatment, the $O_2^{\cdot -}$ and H_2O_2 levels were estimated by cytofluorometry after staining of untreated and treated PC3 cells with 10 μ M HET or 5 μ M DCFH-DA, respectively. The data are expressed as the percentage of control values and are means $\pm$ SE from 3 different experiments. * $p < 0.005$, indicates significant difference between the effects obtained for the cyclopamine and gefitinib alone, as compared to the combined agents.

cence after treatment with these 2 cytotoxic agents as compared to untreated cells. The inhibition of H_2O_2 accumulation by catalase, a scavenger of H_2O_2 , also resulted in a significant reduction of apoptotic death induced by 10 μ M cyclopamine or 5 μ M cyclopamine plus 1 μ M gefitinib, while the specific ceramidase synthase, ynthase, fumonis B1, showed no inhibitory effect at a concentration of 10 μ M (Table I). The broad spectrum caspase inhibitor, Z-VAD-fmk at 50 μ M, also abrogates the PARP cleavage, DNA fragmentation and apoptotic death induced by these agents in PC3 cells (Figs. 5c and d; Table I).

Estimation of the inhibitory effect induced by cyclopamine and gefitinib on the invasive ability of PC3 cells

To estimate the inhibitory effect of the cyclopamine and gefitinib, alone or in combination, on the invasiveness of the PC cells, the invasive potential of untreated or drug-treated PC3 cells was evaluated by their ability to penetrate a Matrigel-invasion chamber. As shown in Figure 7, the treatment of PC3 cells with 2–5 μ M cyclopamine or 0.1 μ M gefitinib significantly inhibited their invasion ability to penetrate the matrix ($p < 0.005$). Moreover, a more marked reduction of the percentage of invasive PC3 cells was observed in the presence of 2 μ M cyclopamine plus 0.1 μ M gefitinib, as compared to the individual drugs ($p < 0.001$).

Discussion

The results of the present investigation revealed that the activation of SHH–GLI-1 and EGF–EGFR pathways together might assume an important role for the growth and survival of PC cells during the progression to highly metastatic states. As a matter of

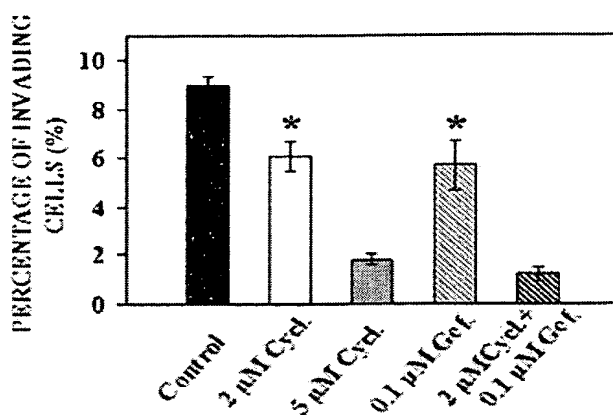


FIGURE 7 – Inhibitory effect of cyclopamine and gefitinib on invasive potential of PC3 cells. The PC3 cells were untreated (control) or pretreated with 2–5 μ M cyclopamine (Cycl.) or 0.1 μ M gefitinib (Gef.), alone or in combination, for 24 hr and during *in vitro* invasion assays, which was performed for an additional 24 hr using BD Biocoat cell invasion chambers as described in Materials and methods. At the end of the assays, the invasive PC3 cells were stained and counted using a hemocytometer by phase-contrast microscopy. The data are expressed as the percentage of invasive cells and are means $\pm$ SE from 3 different experiments. * $p < 0.001$, indicates significant difference between the effects observed for the cyclopamine and gefitinib alone, as compared to the combined agents.

fact, we observed that the metastatic LNCaP-LN3 and androgen-independent LNCaP-C81, DU145 and PC3 cells express higher levels of SHH, PTCH-1, SMO and GLI-1 as well as EGFR as compared to the androgen-responsive LNCaP-C33 and nonmalignant PZ-HPV-7 cells (Fig. 1a). This suggests that the upregulated expression of HH and EGF–EGFR signaling components in PC cells might be a determinant factor associated with the most aggressive and metastatic phenotypes of the tumor cells. In support with this, the recent studies have also revealed significant expression levels of HH signaling components in PC cell lines, including CWR22RV1, LNCaP, DU145 and PC3 cells.^{21,22,24} In particular, a higher expression level of GLI-1 was detected in DU145 and PC3 cells relative to LNCaP cells.^{22,24} Importantly, it has also been observed that the specimens from human advanced or metastatic prostate tumors expressed higher levels of SHH, PTCH-1 and GLI-1 as compared to samples from localized PC and normal tissues or benign PrE cells.^{5,19,22,24} Similarly, our results and many prior studies have also revealed that the EGFR expression level is upregulated in tumoral tissues and PC cell lines, including LNCaP, DU145, PC3 and CWR22RV1, relative to non-malignant PZ-HPV-7 cells and normal and benign tissues.^{31,32,34,35} The enhanced expression and activation of HH and EGFR signaling elements might, therefore, contribute to the initiation and maintenance of PC. In this matter, the benign PrE cells, stably transfected to express GLI-1, showed sustained growth *in vitro* and formed aggressive subcutaneous tumors *in vivo*.²¹ Hence, the aberrant activation of SHH–GLI-1 and EGF–EGFR developmental signaling pathways might be responsible, in part, for the formation and progression of several prostate malignancies, leading to hyperproliferative disorders and cancer in adults.

The activation of the HH signaling pathway by the SHH ligand notably seems to induce the proliferation of PC cells in an autocrine fashion. In this matter, we have observed that the addition of exogenous SHH ligand (SHHNP) induced the stimulation of the proliferation of all PC cells tested. Moreover, the selective SMO inhibitor, cyclopamine, also inhibited the growth in serum-free medium (basal or autocrine growth) and SHHNP-stimulated LNCaP-LN3, LNCaP-C81, DU145 and PC3 cells, while the LNCaP-C33 cells were less sensitive to the inhibitory effect of this

drug. Thus, the concomitant elevated expression of SHH and SMO in the PC cells appears to be related to the activation of the mitotic signaling, leading to a sustained growth of tumor cells. In this matter, it has previously been proposed that an elevated expression level of SMO in PC cells might represent a determinant factor for the activation of a ligand-dependent HH pathway.²¹ Indeed, it has been observed that the overexpression of SMO in benign PrE cells restored the HH pathway activity, which could be blocked by the addition of cyclopamine or anti-SHH 5E1 monoclonal antibody. In addition, since we have observed that the cyclopamine treatment of SHHNP-stimulated PC3 cells was accompanied by downregulating PTCH-1 and GLI-1 expression (Fig. 1b), it appears that elevated PTCH-1 and GLI-1 levels are also required for the mediation of SHH-induced oncogenic effects in PC cells. In support of this, it has been reported that the upregulated expression of SHH in diverse cancer cell types was accompanied by an increasing expression rate of PTCH-1 receptor and GLI-1 effector and the transactivation of numerous mitotic genes.^{5,14,49} Moreover, the cyclopamine treatment has been observed to suppress the GLI-1 expression in LNCaP cells.²² Some previous studies have also indicated that the inhibition of SHH signaling in numerous other cancer epithelial cell types by using cyclopamine resulted in the downregulation of GLI-1 and PTCH-1 expression or activities concomitant with an arrest of the growth of tumor cells *in vitro* and *in vivo*.^{11,12,18,37} Conversely, the inhibition of SHH signaling by anti-SHH antibody or cyclopamine has also been reported to result in the downregulation of PTCH-1 and GLI-1 expression levels that was accompanied by severe defects in prostate ductal morphogenesis.^{1,4,6,9} Together, these observations suggest that the changes in the expression levels and activities of SHH-PTCH-1-GLI-1 signaling components (prevalent during prostate development) might also be manifested in the adult prostate, because of diverse pathological processes occurring in PrE cells. This could lead to the initiation and development of PC.

Since the results of the present study indicated that the cyclopamine and gefitinib, alone or in combination, also inhibit SHHNP-, EGF- and serum-stimulated growth of PC cells, it appears that the mechanistic interactions involving common signaling elements between HH signaling and other growth factor pathways might contribute to the PC cell growth. In support of this, the results indicated that the inhibitory effect induced by cyclopamine or gefitinib on the SHHNP- and EGF-stimulated growth of PC3 cells is mediated *via* an arrest of the cells in the G1 phase, concomitant with a decrease of the cell population in the S and G2M phases (Fig. 3). In this matter, it has been reported that the activation of SHH-GLI-1 signaling, which leads to the proliferation of mammalian cells, might be mediated, at least, in part, through the upregulated expression of the cell cycle regulators, including the cyclins.^{14,50} Hence, the blockade of HH signaling by cyclopamine might counteract numerous mitogenic cascades induced by other growth factors, including EGF in PC cells through the negative modulation of the cell cycle regulators. Previous works also indicated that the cyclopamine treatment of LNCaP, DU145 and PC3 as well as the primary prostate tumors was accompanied with a significant decrease of DNA synthesis as indicated by a significant reduction of BrdU incorporation.^{22,24} Moreover, the cyclopamine alone has been observed to induce a significant inhibition of the growth of CWR22RV1, DU145 and PC3 cells *in vitro* and a complete regression within 20–24 days of cyclopamine treatment of the xenograft tumors established by PC3 and 22RV1 cells in athymic mice.²¹ Similarly, numerous investigations also indicated that the blockade of the EGF-EGFR transduction system by using a selective EGFR inhibitor, such as gefitinib or anti-EGFR antibody, resulted in an inhibition of the proliferation of MDAPCa, LNCaP, DU145 and PC3 PC cell lines and primary cultures as well as in the regression of prostate tumor growth established in mice *in vitro*.^{35,38–41,51–54} The growth inhibitory effect induced by blocking EGFR in PC cells appears notably to be mediated by upregulating p27^{KIP1} levels, which, in turn, inhibits the mitotic effects

induced through cyclin-CDK complexes. More specifically, since we have observed that the simultaneous blockade of HH and EGF-EGFR pathways by cyclopamine and gefitinib resulted in an arrest of the growth of PC3 cells, it appears that these 2 cascades might act cooperatively for the proliferation of metastatic PC cells. As a matter of fact, the results indicated that the cyclopamine treatment of SHHNP-stimulated PC3 cells resulted in a downregulation of EGFR expression as compared to untreated cells (Fig. 1b). In this context, it has been reported that SHH can act in synergy with EGF to promote neurosphere growth during the brain development, as indicated by BrdU incorporation.⁵⁵ Moreover, the RT-PCR analysis of neocortical tissue has also revealed that *in vivo* cyclopamine treatment results in a decreased GLI-1 expression, which was also accompanied by downregulating the EGFR expression level.⁵⁵ In the same way, the stimulation of the HH cascade has been observed in *Drosophila* to increase EGFR (DER) signaling by upregulating the expression of the EGFR ligand designated vein (vn), of which the mitogenic effect was essential for normal development.^{56–58} Importantly, it has also been observed that the uncontrolled activation of SHH-GLI-1 signaling in the precursor cells was associated with the hyperproliferation and initiation of tumorigenesis of specific tumor types, including the brain and skin.^{5,8,11,12} Although it still remains unclear whether the tumors derive from the aberrant activation of SHH-GLI-1 and EGF-EGFR in stem cells or progenitor cells or more differentiated cells, it appears that certain molecular interplay between these mitogenic cascades, which are prevalent during embryogenesis and morphogenesis in vertebrates and invertebrates, might also be manifested in certain cancer epithelial cells overexpressing these signaling elements.

Of therapeutic interest, the results revealed that the cyclopamine, alone or at lower concentrations with gefitinib, induces a massive rate of apoptotic death on the metastatic LNCaP-LN3 cells and androgen-independent LNCaP-C81, DU145 and PC3 cells, while LNCaP-C33 cells were less responsive to cytotoxic effects of these drugs (Figs. 4–6 and Table I). Although the reasons responsible for the lower sensitivity of LNCaP-C33 to cyclopamine treatment as compared to other PC cells tested is not known, it is likely that distinct signaling elements might contribute to the survival of these cancer cells. Moreover, the data have also revealed that the apoptotic effect induced by cyclopamine and gefitinib in PC3 cells might be mediated, at least, in part, through the mitochondrial pathway, involving the depolarization of the mitochondrial membrane, the release of the cytochrome *c* and ROS into cytosol, the activation of caspase cascades and the PARP cleavage, which ultimately leads to the DNA fragmentation. This supports the previous studies that indicated that cyclopamine and gefitinib alone induced the apoptotic death of PC cells.^{24,40,41,54} In particular, the results from the TUNEL assay analyses indicated that the cyclopamine induced apoptosis in parental PC3 cells, while the transformed PC3 cells overexpressing GLI-1 did not undergo apoptosis.²⁴ Therefore, the SMO inhibition by cyclopamine, which leads to GLI-1 and EGFR downregulation (Fig. 1b), might contribute to triggering apoptotic cell death. Moreover, since the superoxide anions also appears to reduce the EGFR protein expression level in PC3 cells,⁵⁹ this suggests that cyclopamine and gefitinib-induced O₂⁻ production might also be responsible, in part, for the antiproliferative and cytotoxic effects induced by these drugs in PC3 cells (Fig. 6). The ability of cyclopamine plus gefitinib to induce the massive cell death of PC suggests that the SHH and EGF-EGFR pathways might also act in conjunction for the cell survival of prostate tumor cells. Consistent with this, the EGF has also been reported to inhibit cyclopamine-induced apoptotic death of the mouse basal cell carcinoma cell line ASZ001.³⁷ Therefore, since the cyclopamine appears to selectively induce apoptosis in tumor cells without adverse effects on normal tissues *in vivo*,⁶⁰ this suggests the potential to use cyclopamine, alone or in combination at lower doses with EGFR inhibitor, in adjuvant therapy for the prevention of PC progression.

In addition, we have also observed that HH and EGF-EGFR signaling pathways might cooperate to enhance the invasive properties of PC cells. Indeed, the results have revealed that the inhibition of HH and EGF-EGFR signaling by cyclopamine plus gefitinib induced a greater inhibition of the invasiveness of PC3 cells observed *in vitro* assays as drugs alone (Fig. 7). In support with this, the upregulation of SHH and EGF-EGFR signaling has also been associated with an increased invasive and metastatic potential of PC cells, while the blockade of these mitogenic cascades by cyclopamine, anti-EGF antibody or EGFR inhibitor, including gefitinib, reduced the invasive abilities observed for PC cells *in vitro* and *in vivo*.^{21,27,29,31-33,61,62} In particular, it has been reported that the cyclopamine treatment *in vitro* inhibited the invasive abilities of PC3 and DU145 as well as highly metastatic AT6.3 PC cells.²¹ Interestingly, a recent study carried out with curcumin has also revealed that the inhibition of the EGFR tyrosine kinase activity by this agent was accompanied by a reduction of osteomimetic properties of C4-2B, a highly metastatic derivative of LNCaP, with properties that appear to be related to their

ability to establish osseous metastases.⁶³ Then, these findings indicate that the inhibition of SHH-GLI-1 and EGF-EGFR signaling by cyclopamine and gefitinib could also counteract the invasiveness of PC cells *in vivo*.

Taken together, the results of the present study revealed that the simultaneous blockade of the SHH-GLI-1 and EGF-EGFR pathways, by using cyclopamine and EGFR inhibitor, which leads to an arrest of growth as well as an increased rate of apoptotic death and decreased invasive abilities of PC cells, might represent a novel therapeutic approach to counteract the progression of localized prostate tumors to advanced and metastatic forms of PC, which are incurable by current conventional therapies.

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Research

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Activation of the hedgehog pathway in advanced prostate cancer

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Abstract

Background: The hedgehog pathway plays a critical role in the development of prostate. However, the role of the hedgehog pathway in prostate cancer is not clear. Prostate cancer is the second most prevalent cause of cancer death in American men. Therefore, identification of novel therapeutic targets for prostate cancer has significant clinical implications.

Results: Here we report that activation of the hedgehog pathway occurs frequently in advanced human prostate cancer. We find that high levels of hedgehog target genes, PTCH1 and hedgehog-interacting protein (HIP), are detected in over 70% of prostate tumors with Gleason scores 8–10, but in only 22% of tumors with Gleason scores 3–6. Furthermore, four available metastatic tumors all have high expression of PTCH1 and HIP. To identify the mechanism of the hedgehog signaling activation, we examine expression of Su(Fu) protein, a negative regulator of the hedgehog pathway. We find that Su(Fu) protein is undetectable in 11 of 27 PTCH1 positive tumors, two of them contain somatic loss-of-function mutations of Su(Fu). Furthermore, expression of sonic hedgehog protein is detected in majority of PTCH1 positive tumors (24 out of 27). High levels of hedgehog target genes are also detected in four prostate cancer cell lines (TSU, DU145, LN-Cap and PC3). We demonstrate that inhibition of hedgehog signaling by smoothened antagonist, cyclopamine, suppresses hedgehog signaling, down-regulates cell invasiveness and induces apoptosis. In addition, cancer cells expressing Gli1 under the CMV promoter are resistant to cyclopamine-mediated apoptosis. All these data suggest a significant role of the hedgehog pathway for cellular functions of prostate cancer cells.

Conclusion: Our data indicate that activation of the hedgehog pathway, through loss of Su(Fu) or overexpression of sonic hedgehog, may involve tumor progression and metastases of prostate cancer. Thus, targeted inhibition of hedgehog signaling may have significant implications of prostate cancer therapeutics.

Background

The *hedgehog* (Hh) pathway plays a critical role in embryonic development and tissue polarity [1]. Secreted Hh molecules bind to the receptor *patched* (PTC-PTCH1, PTCH2), thereby alleviating PTC-mediated suppression of *smoothened* (SMO), a putative seven-transmembrane protein. SMO signaling triggers a cascade of intracellular events, leading to activation of the pathway through Gli-dependent transcription [2]. The *hedgehog* receptor (PTCH1) is also a target gene of this pathway, which forms a negative feedback mechanism to maintain the pathway activity at an appropriate level in a given cell. Activation of Hh signaling through loss-of-function somatic mutations of PTCH1 in human basal cell carcinomas (BCCs) disrupts this feedback regulation, leading to uncontrolled SMO signaling. Activating mutations of SMO in BCCs, on the other hand, are resistant to PTCH1-mediated inhibition, leading to an outcome similar to PTCH1 inactivation [3-6]. More recently, abnormal activation of the sonic hedgehog pathway, through over-expression of sonic hedgehog, has been implicated in the development of subsets of medulloblastomas, small cell lung cancer and gastrointestinal tract (GI) cancers [7-10].

Development of prostate requires hedgehog signaling. Although the initial formation of prostate buds does not require sonic hedgehog signaling (*shh*), *shh* is critical for maintaining appropriate prostate growth, proliferation and tissue polarity [11-14]. In the adult prostate, however, the activity of the hedgehog pathway is quite low. It remains to be tested whether this hedgehog pathway is activated during development of prostate cancer, the second most prevalent cause of cancer death in American men. Activation of the hedgehog pathway is often indicated by elevated levels of PTCH1 and HIP. In addition to PTCH1 mutation, SMO activation and hedgehog over-expression, loss of *Su(Fu)* can result in activation of the hedgehog pathway. In the human, the *Su(Fu)* gene is localized at chromosome 10q24, a region with LOH in several types of cancer including prostate cancer, lung cancer, breast cancer and medulloblastomas [15,16]. As a negative regulator of the hedgehog pathway, *Su(Fu)* inhibits the function of Gli molecules, leading to inactivation of this pathway [17-19]. *Su(Fu)* is also reported to affect beta-catenin function [20]. In addition, over-expression of sonic hedgehog is shown to be involved in the development of GI cancers [9,10]. (Here we report our findings that activation of the hedgehog pathway occurs frequently in advanced prostate cancers, possibly through loss of *Su(Fu)* protein or over-expression of sonic hedgehog.

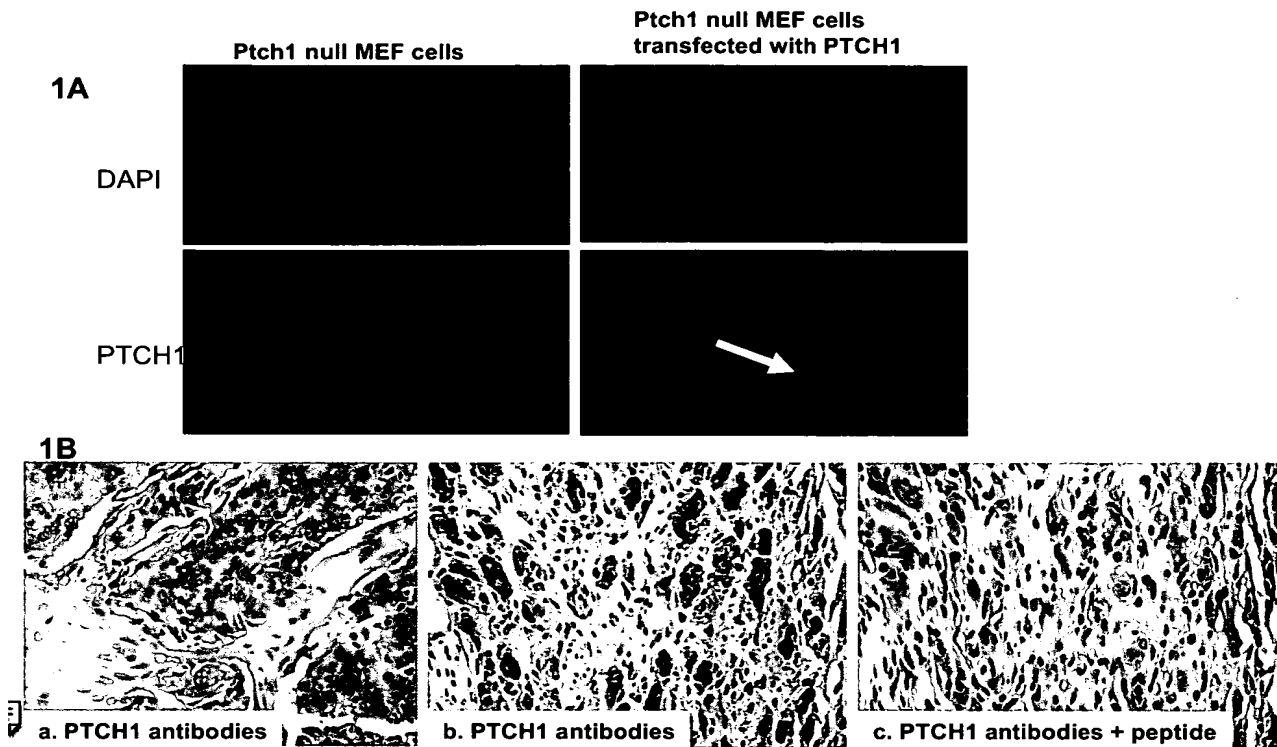
Results

Elevated expression of hedgehog target genes in prostate cancer specimens

As an important regulator of tissue polarity, active hedgehog signaling is required for ductal morphogenesis and proliferation during prostate development [11-14]. The adult prostate, on the other hand, does not contain active hedgehog signaling. Because hedgehog signaling is an important regulator for epithelial-mesenchymal interaction, an event critical during prostate cancer development, we examined whether the hedgehog-signaling pathway is activated in prostate cancer.

Activation of hedgehog signaling causes elevated expression of target genes PTCH1 and HIP. Thus, increased protein expression of PTCH1 and HIP indicates activation of the hedgehog pathway. Using PTCH1 antibodies [10], we examined 59 prostate cancer samples for hedgehog signaling activation (see Table 1, Additional file 1 for details). We first tested the specificity of the PTCH1 antibodies in MEF cells. *Ptch1* null MEF cells have no active *Ptch1* gene, thus should not have positive staining with PTCH1 antibodies. Indeed, no staining was seen in *Ptch1* null MEF cells (Fig. 1A). After transfection of PTCH1 expressing plasmid, transfected cells showed positive staining (Fig. 1A), indicating that the PTCH1 antibodies are specific to PTCH1. Furthermore, PTCH1 immunohistochemistry was abolished after addition of the specific peptide, from which the antibodies were raised (Fig. 1B,1c). We found that percentage of PTCH1 positive staining tumors increased in high grade tumors (Table 1, Additional file 1). In prostate cancers with Gleason scores 3-6, 4 out of 18 specimens were positive for PTCH1 (22%), whereas 16 out of 22 undifferentiated carcinomas (Gleason Scores of 8-10) expressed PTCH1 (73%, see Table 1, Additional file 1), suggesting that the hedgehog pathway is frequently activated in advanced prostate cancer. To confirm this data, we found that all four available metastatic prostate cancer specimens were all positive for PTCH1 staining.

To further confirm our data, we detected HIP protein expression, another marker of the hedgehog signaling activation. After transfection of HIP expressing plasmid into 293 cells, HIP antibodies recognize a single band around 75 KD (Fig. 3A), and an endogenous HIP protein with a similar size was also detected in two cancer tissues, in which hedgehog signaling is known to be activated (Fig. 3B and data not shown here). In contrast, the matched normal tissue did not express detectable HIP. Thus, HIP expression appears to be a good marker for hedgehog signaling activation. Immunohistochemistry with HIP antibodies in prostate cancer specimens revealed a similar pattern to prostate specific antigen (PSA) and PTCH1 (Fig. 3C and Table 1, Additional file 1), further confirming that hedgehog pathway is activated in

**Figure 1**

Detection of PTCH1 expression in prostate cancers. Protein expression of PTCH1 was detected by immunostaining. PTCH1 antibodies (Santa Cruz Biotechnology Cat# 9149) were tested in *Ptch1*^{-/-} null MEF cells (A). While *Ptch1*^{-/-} null MEF cells had no positive fluorescent staining with PTCH1 antibodies, transfection of PTCH1 expressing plasmid lead to positive staining (green, indicated by an arrow, 400×). Immunohistochemistry of prostate cancer specimens with PTCH1 gave negative (B-a, 200×) or positive (Red in B-b, 200×) signals. When PTCH1 antibodies were pre-incubated with the very peptide for raising the antibodies, no positive signals could be observed (B-c).

advanced prostate cancers. Thus the hedgehog pathway appears to be frequently activated in advanced or metastatic prostate cancers.

Altered expression of Su(Fu) and Shh in prostate cancer specimens

There are several mechanisms by which the hedgehog pathway in these prostate tumors can be activated, including loss of Su(Fu) or over-expression of hedgehog [6-10]. The Su(Fu) gene is localized at 10q24, a region with a frequent LOH in prostate cancer [15,16,18]. Mutations of Su(Fu) have been reported in other human cancers [6]. To test whether loss of Su(Fu) function is responsible for hedgehog signaling activation, we examined expression of

Su(Fu) protein in these prostate cancer specimens. The antibodies of Su(Fu) recognize a single band at 52-kD in Western blotting analyses (Fig. 4A), which was reduced following treatment with Su(Fu) SiRNA (Fig. 4B), indicating the specificity of the antibodies. Furthermore, addition of the peptide, from which the antibodies were raised, prevented the antibody binding, further confirming the specificity of our Su(Fu) antibodies (data not shown). Of the 16 PTCH1 positive prostate cancer specimens with Gleason scores 8-10, 9 have no detectable Su(Fu) protein (Fig. 4C,4D,4E and Table 1, Additional file 1). In total, 11 of 27 PTCH1 positive prostate cancer specimens have no detectable Su(Fu) protein. Prostate cancers with low Gleason scores, however, frequently have

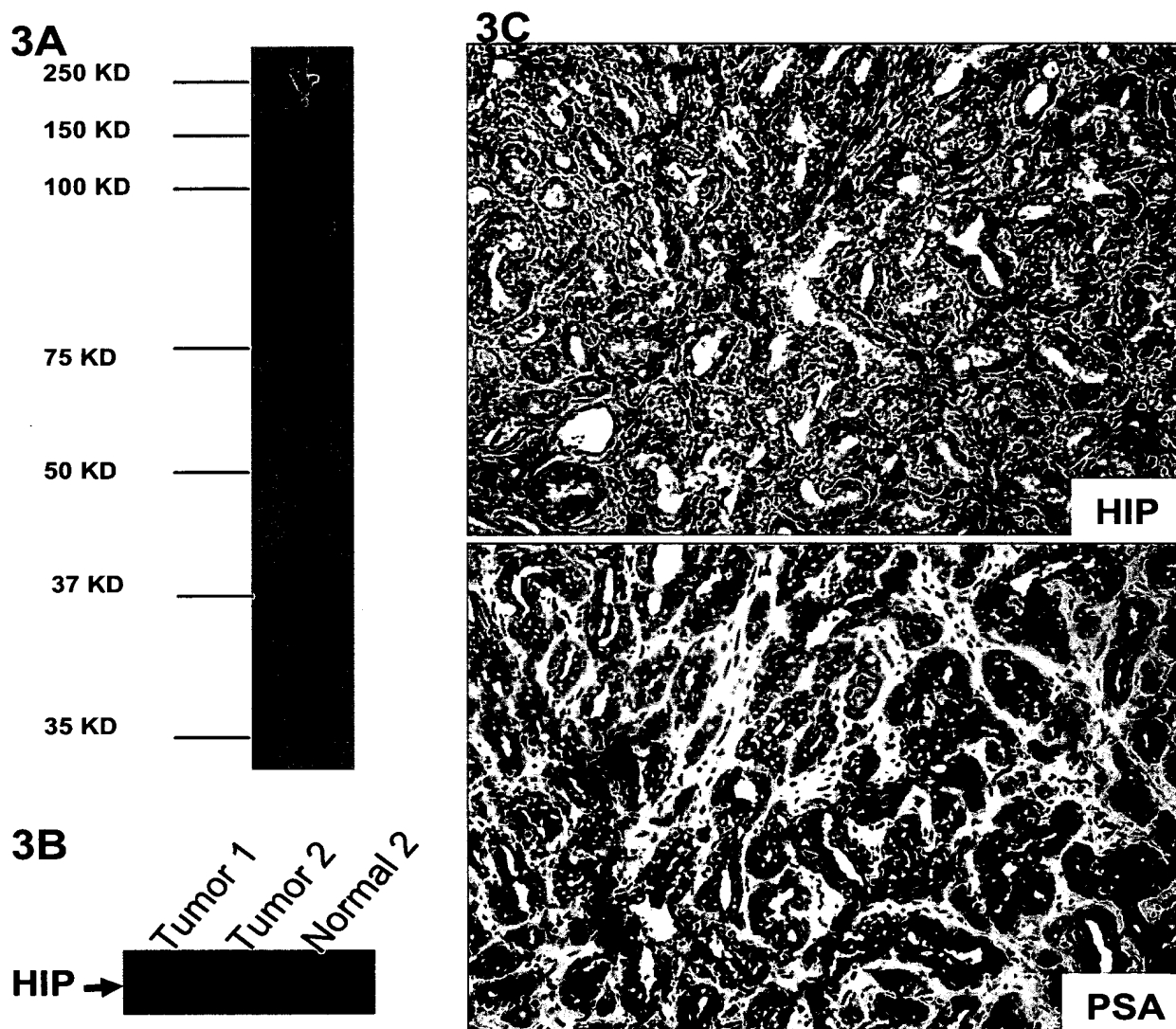


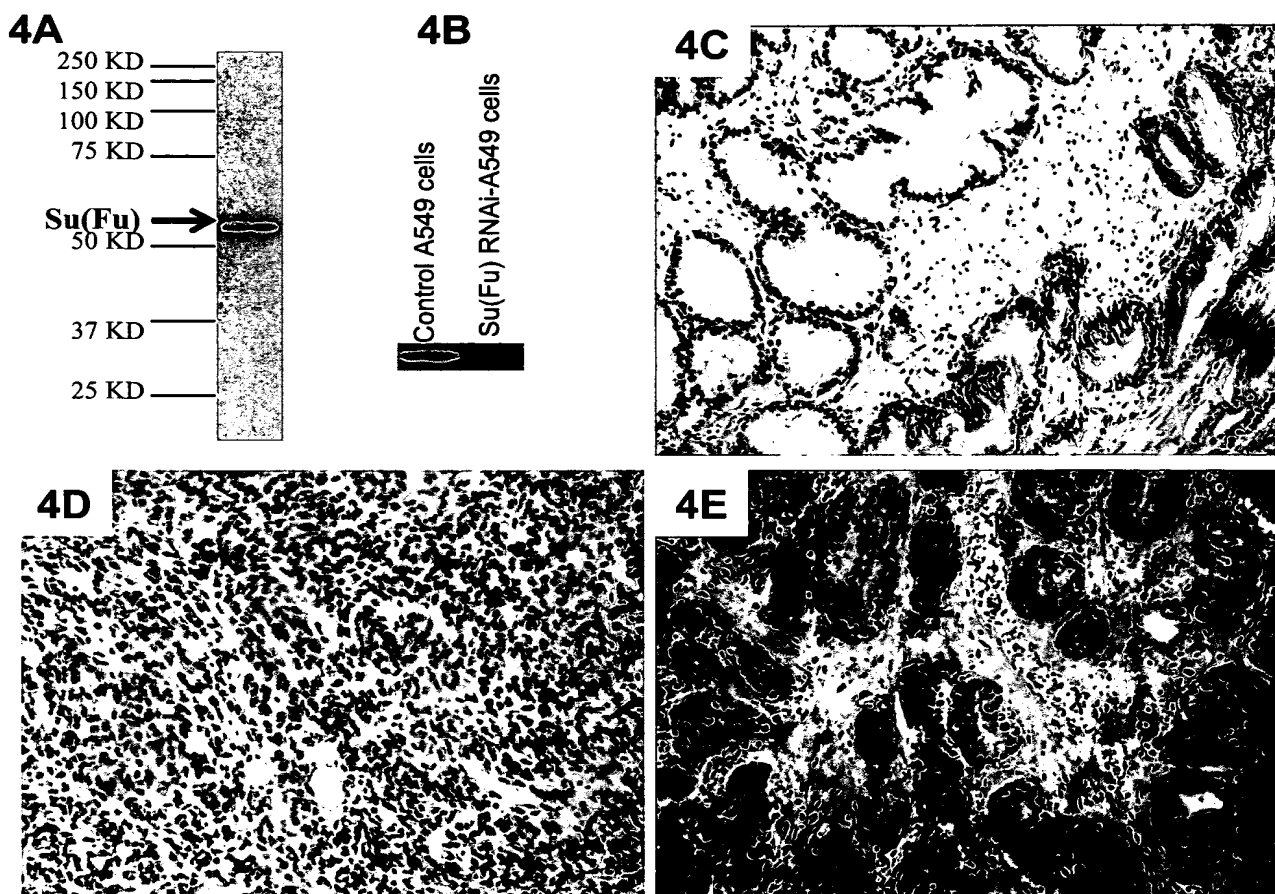
Figure 3

Detection of HIP in human cancer specimens. By Western blotting, HIP antibodies (R&D systems Cat# AF1568) recognized one band between 75 and 100 KD (A). Expression of endogenous HIP was detected in two GI cancer tissues, which were known to contain activated hedgehog signaling (data not shown here), but not in the matched normal tissue (B). Immunohisto-staining of HIP I prostate cancer showed a similar pattern to PSA (C, 200×)

detectable Su(Fu) protein (see Table 1, Additional file 1), suggesting that loss of Su(Fu) protein may be associated with prostate cancer progression.

To confirm the immunohistochemistry data, we performed immunoblotting analyses using several dissected

TURP (Transurethral resection of the prostate) specimens in which tumor portion can be as high as 70% of the tissue mass. Prostatectomy specimens (most of our tumors), however, often contain a small percentage (5–10%) of tumor tissue and are therefore not suitable for Western blotting or real-time PCR analyses. As shown in Fig. 5A,

**Figure 4**

Detection of Su(Fu) in prostate cancer specimens. Su(Fu) antibodies (Santa Cruz Biotechnology Cat# 10933) recognized only one single band (54-Kd) in D283 cells (A). Following treatment of a specific SiRNA of Su(Fu), the endogenous Su(Fu) band was greatly reduced (B). Immunohistostaining with Su(Fu) antibodies in prostate cancer specimens revealed positive (C, in red, 200 $\times$), negative (D, 200 $\times$) or weak staining (E, red, 200 $\times$).

two tumors (PC48 and PC51) had no detectable Su(Fu) protein, which are consistent with our immunohistostaining, suggesting loss of Su(Fu) may be responsible for hedgehog pathway activation in these tumors. The matched normal tissues, however, retained expression of Su(Fu), indicating that alteration of Su(Fu) is a somatic event. Sequence analyses of these two tumors revealed genetic mutations in *Su(Fu)*, which are predicted to create STOP codons in the coding sequence (Fig. 5B and Table 1, Additional file 1). In PC48, a homozygous deletion of A1315 was detected, which results in a STOP codon at +1318 bp (Fig. 5B). In PC51, we detected two types of mutations, one with a deletion of C255, which results in

a STOP codon at +294 bp whereas another with a deletion of C198, create a STOP codon (Picture not shown here, see Table 1, Additional file 1). These mutations were confirmed with 6 independent clones from two separate experiments, which exclude the possibility of PCR errors. No mutations were detected from the matched benign tissues, indicating the somatic nature of the mutations. Real-time PCR analyses indicated that target genes of the hedgehog pathway, PTCH1 and Gli1, were all elevated in these tumors (Fig. 5C), confirming activation of the hedgehog pathway in these tumors. Thus, Su(Fu) inactivation appears to contribute to activation of hedgehog signaling in these prostate tumors.

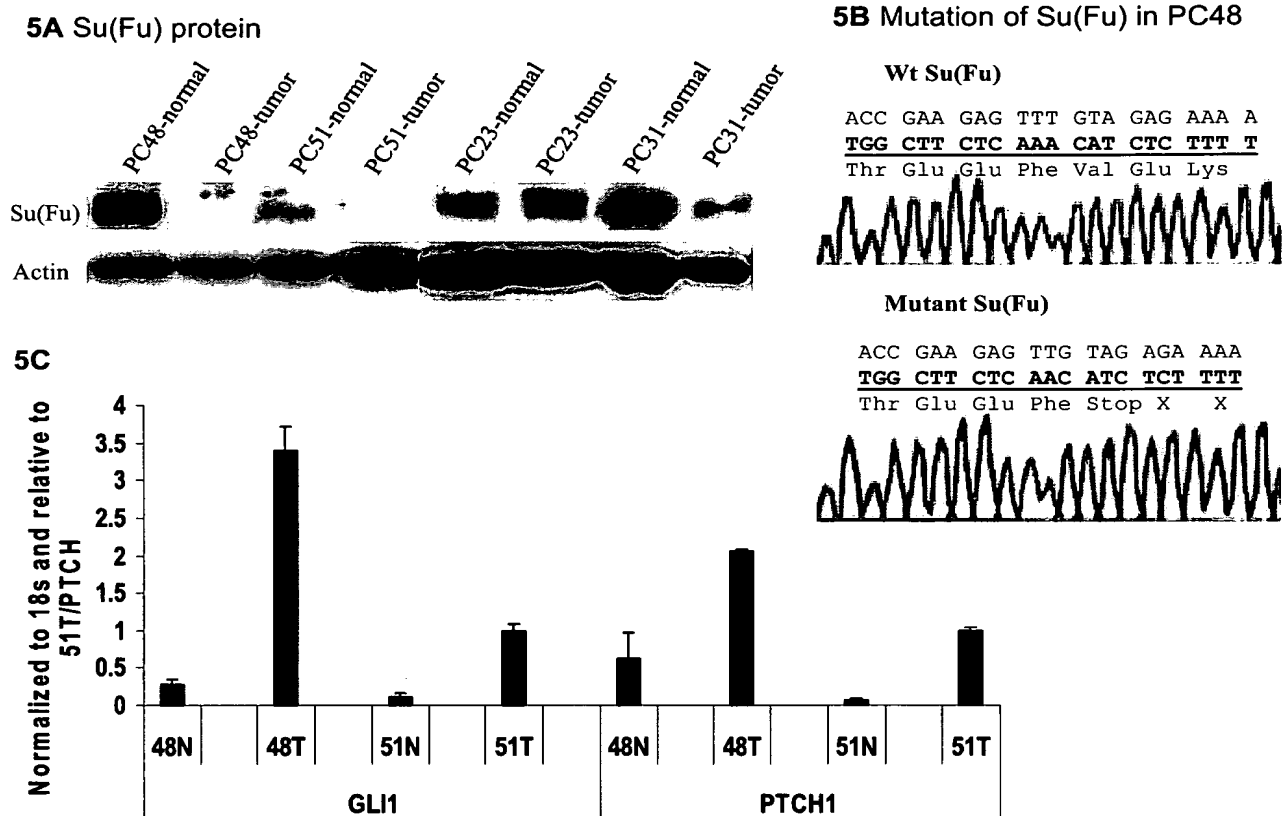


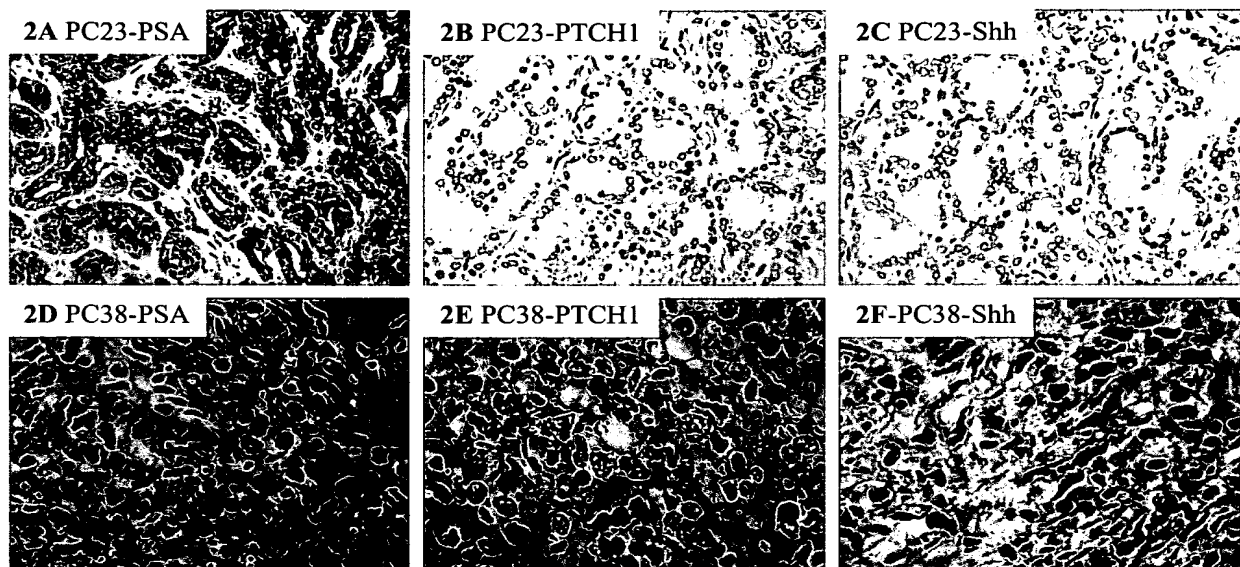
Figure 5
Inactivation of Su(Fu) in prostate cancer. Two TURP (Transurethral resection of the prostate) tumors with loss of Su(Fu) expression were confirmed by Western blotting (A). One mutation of Su(Fu) found in prostate cancer PC48 is shown in B, which is predicted to create a STOP codon in the Su(Fu) coding sequence +1318. The levels of Gli1 and PTCH1 transcripts in prostate tissues were detected by real-time PCR (see methods for details) (C). Tumor tissues had higher levels of the target gene transcripts.

For tumors with high level of PTCH1 expression, but no changes in Su(Fu) protein expression, we examined expression of sonic hedgehog. It is reported that expression of hedgehog may be responsible for hedgehog signaling activation in lung cancer and GI cancers. Immunohistostaining with sonic hedgehog antibodies indicate that (sonic hedgehog is highly expressed in 24 of 27 advanced prostate tumors with elevated expression of PTCH1) and HIP (see Fig. 2 and Table 1, Additional file 1). Thus, activation of the hedgehog pathway, as indicated by elevated PTCH1 and HIP expression, is associated with loss of Su(Fu) expression or elevated hedgehog expression.

The role for activated hedgehog signaling for cellular functions of prostate cancer

To demonstrate the role of hedgehog pathway in prostate cancer, we screen five available cell lines for the expression of Gli1, PTCH1 and HIP. TSU, LNCap, Du145 and PC3 are prostate cancer cell lines whereas RWPE-1 is a prostate epithelial cell line. We found that the (hedgehog target genes were significantly elevated in all cancer cell lines (Fig. 6A). Thus, we predicted that inhibition of the hedgehog pathway by smoothened antagonist, cyclopamine, would suppress cell proliferation and cell invasiveness.

Following treatment with 5 μ M cyclopamine in PC3 cells, expression of hedgehog target genes were dramatically inhibited (data not shown here), which was accompanied

**Figure 2**

Co-expression of PTCH1, PSA and Shh in prostate cancer specimens. Immunohistochemistry of prostate cancer specimens with PSA was used to confirm the cancer region. Positive staining was in red. Positive staining patterns of PTCH1 and Shh antibodies (Santa Cruz Biotechnology Cat# 9024) were similar to that of PSA staining. PC23 (A-C) was from tumors with Gleason score 7 (200 $\times$). PC38 (D-F) was a tumor from Gleason score 10 (400 $\times$) (see Table 1, Additional file 1 for details).

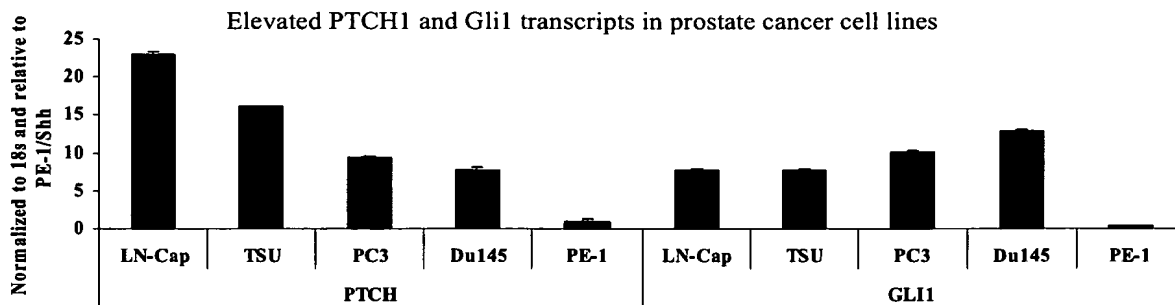
with a significant reduction of BrdU positive cells (see Fig. 6B for details). This effect is specific because addition of tomatidine, a non specific compound with a similar structure to cyclopamine, had no effects on either target gene expression or DNA synthesis (indicated by BrdU labeling in Fig. 6B and 6C). The prostate epithelial RWPE-1 cells which have no activated hedgehog signaling, on the other hand, were not sensitive to cyclopamine (data not shown here), indicating that cyclopamine specifically affects cells with elevated hedgehog signaling. LN-CAP, Du145 and TSU cells, like PC3 cells were also sensitive to cyclopamine treatment (Fig. 6C).

Prostate cancer progression is accompanied by increased cell invasiveness. Because the hedgehog signaling activation occurs frequently in advanced prostate cancer, we examined if inhibition of the hedgehog signaling can reduce cell invasiveness. Using BD Bio-coat cell invasion chambers, we found that treatment of cyclopamine in PC3 cells reduced the percentage of invasive cells by 70% (Fig. 7A). Similar data were also observed in Du145, LN-CAP and TSU cells (Fig. 7B). Under the same condition,

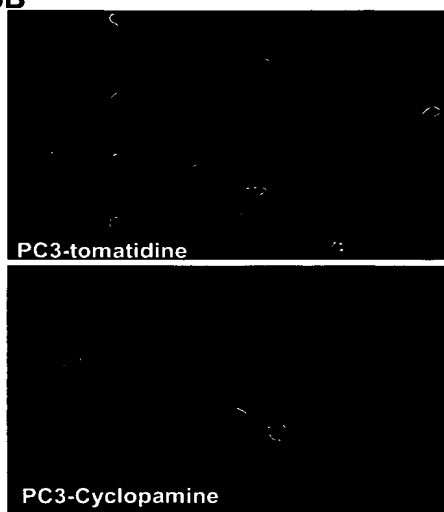
RWPE-1 cells were not very invasive. Thus, hedgehog signaling activation regulates both cell proliferation as well as cell invasiveness of prostate cancer cells.

It has been shown that cyclopamine induced apoptosis in cancer cells with activated hedgehog signaling [21]. We have shown that Gli1 down-regulation is necessary for cyclopamine-mediated apoptosis in basal cell carcinoma cells [21]. To test the significant role of Gli1, the downstream effector and the target gene of the hedgehog pathway, in cyclopamine-mediated apoptosis, we first transfected Gli1 expressing plasmid in to PC3 cells, and then treated the cells with 5 μ M cyclopamine for 36 h. Since Gli1 is expressed under the control of the CMV promoter, we predicted that ectopic Gli1-expressing cells should be resistant to apoptosis, which is detected by TUNEL staining. As shown in Fig. 8, we found that all Gli1 positive cells (n = 500) were TUNEL negative, supporting our hypothesis that down-regulation of Gli1 may be an important mechanism by which cyclopamine mediates apoptosis in prostate cancer cells with activated hedgehog signaling.

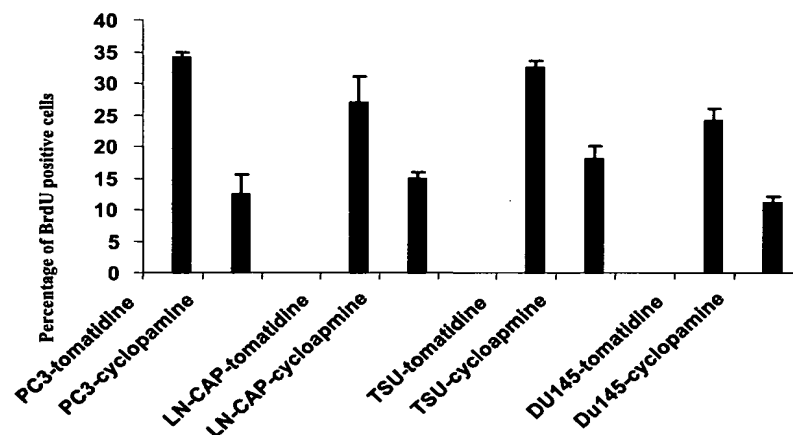
6A



6B



6C

**Figure 6**

Cellular functions of the hedgehog pathway in prostate cancer cells. Expression of hedgehog target genes, PTCH1 and Gli1, were detected by real-time PCR (A). DNA synthesis was detected by BrdU labeling (B). Over 1000 cells were counted under fluorescent microscope for the percentage of BrdU positive cells, and the experiment was repeated twice (C).

All these data indicate that the hedgehog pathway is activated in advanced prostate cancers, as indicated by high expression of PTCH1 and HIP. Our results also indicate that hedgehog signaling is required for cell proliferation and cell invasion of prostate cancer cells. Thus, targeted inhibition of the hedgehog pathway may be effective in future prostate cancer therapeutics.

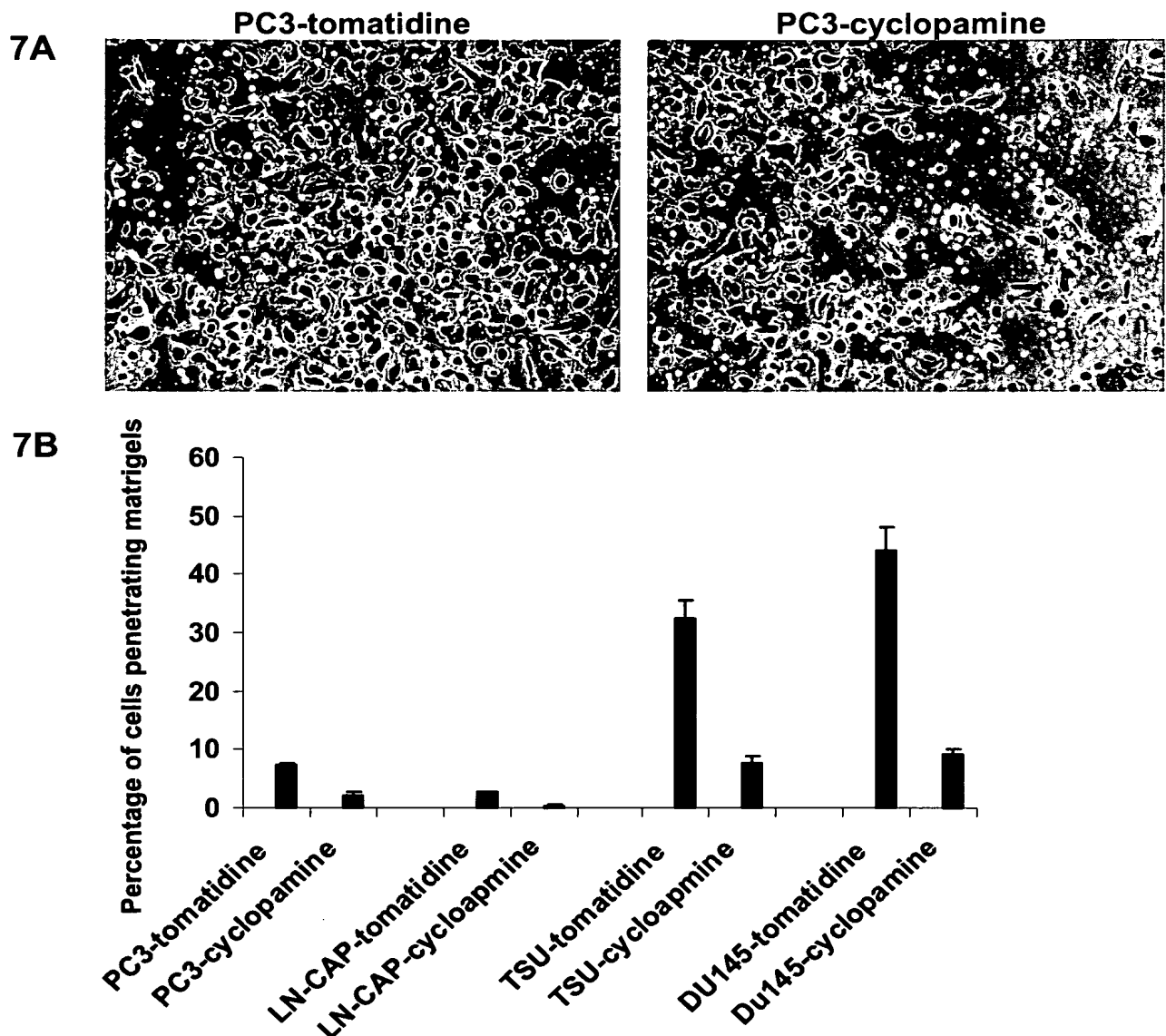
Discussion

Hedgehog signaling pathway regulates cell proliferation, tissue polarity and cell differentiation during normal development. Abnormal signaling of this pathway has been reported in a variety of human cancers, including basal cell carcinomas, medulloblastomas, small cell lung cancer and GI cancers [3,4,6-10,22,23]. Our findings in

this report indicate a role of the sonic hedgehog pathway in prostate cancer. We detected a high expression of hedgehog target genes, PTCH1 and HIP, in advanced or metastatic prostate cancers. In contrast, only 22% of prostate tumors with Gleason scores 3-6 have elevated expression of PTCH1 and HIP. While our manuscript is being reviewed, three independent groups have recently reported similar results [24-26]. Thus, the hedgehog signaling pathway is frequently activated in advanced or metastatic prostate cancers.

Alterations of genes in the hedgehog pathway in prostate cancer

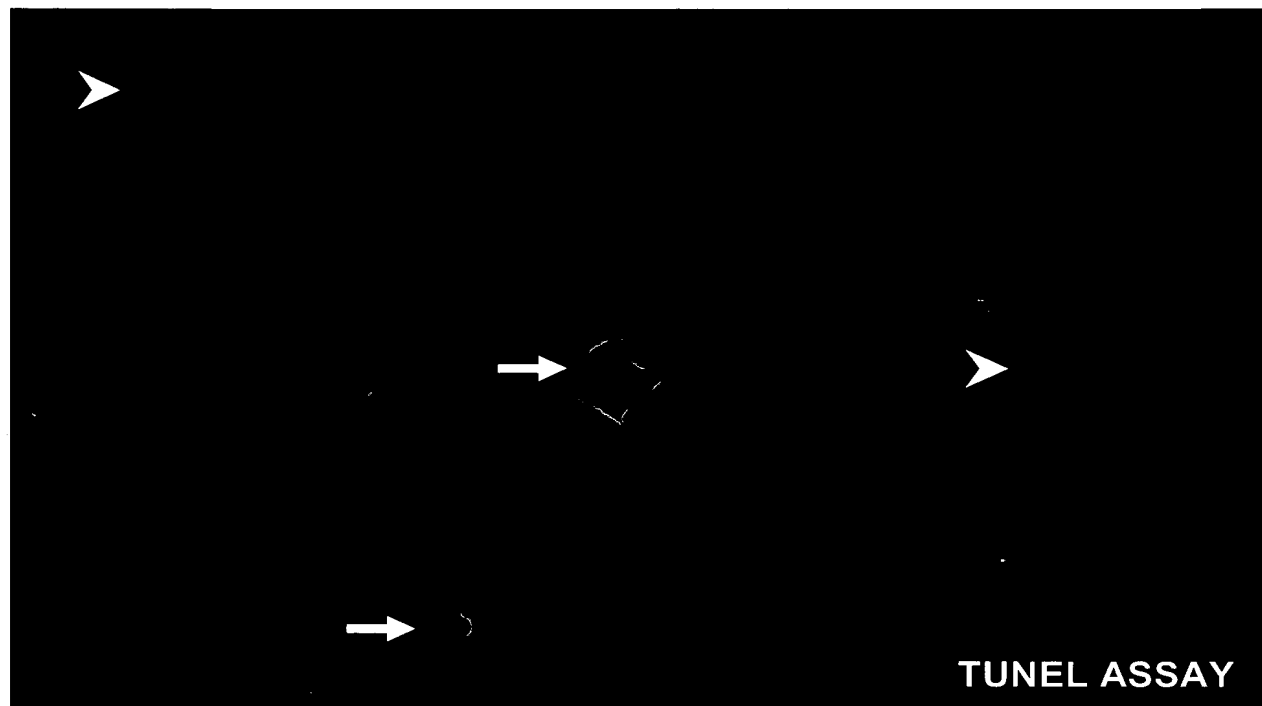
In our studies, we found that some prostate tumors had no detectable Su(Fu) protein expression while others

**Figure 7**

Effects of cyclopamine on cell invasiveness of prostate cancer cells. Cell invasion assay of prostate cancer cells was performed using BD Bio-coat cell invasion chambers (A). The rate of cell invasion was calculated by dividing cell numbers penetrated the matrigels by the number of cell in the control chambers (without matrigels) (B).

contained high levels of Shh protein expression. We further identified inactivated mutations of *Su(Fu)* in two prostate cancers. In addition to inactivated mutations in the coding region, *Su(Fu)* may be inactivated through

promoter methylation. The heterogeneous nature of prostate cancer makes it difficult to screen prostate cancer specimens for *Su(Fu)* mutations since the tumor content is often less than 5% of the specimens. Future improve-

**Figure 8**

Cyclopamine induces apoptosis in prostate cancer cells. Cyclopamine-mediated apoptosis in prostate cancer cells was analyzed by TUNEL assay. TUNEL positive cells were indicated by arrowheads. Cells with expression of Gli1 under the CMV promoter (indicated by the arrows) did not undergo apoptosis (n = 500).

ment can be achieved using microdissection techniques for collecting pure population of tumor cells in gene mutation analysis.

Since all available prostate cancer cell lines express Su(Fu) at a high level, the role of Su(Fu) on cellular functions of prostate cancer cannot be investigated in these cells. It appears that over-expression of sonic hedgehog may be responsible for hedgehog signaling activation in these cell lines [our unpublished data and [24-26]]. After screening over 30 human cancer cell lines, we identified non-prostate cancer cell line with elevated hedgehog target genes and no detectable Su(Fu) expression (data not shown here). The growth suppression effects of Su(Fu) was demonstrated in this cell line, in which Su(Fu) expression down-regulated hedgehog target genes, inhibited DNA synthesis and cell growth (data not shown here). Thus, inactivation of Su(Fu) can contribute to active hedgehog signaling in prostate cancer.

Su(Fu) is reported to affect β -catenin signaling [27,28]. We analyzed expression of β -catenin and E-cadherin in our prostate cancer array and detected cytoplasmic distribution of E-cadherin and β -catenin only in PC51 (data not shown), indicating that Su(Fu) may be able to affect both the wnt pathway and the hedgehog pathway in prostate cancer.

In addition to Su(Fu) inactivation, over-expression of Shh is another mechanism by which the hedgehog pathway is activated in cancer [7-10]. We noticed that sonic hedgehog expression varies from tumor to tumor, which may be resulted from the heterogeneity of prostate cancer. Our immunohistostaining also revealed that three tumors (PC14, PC20 and PC36) expressed PTCH1 and HIP at high levels, but had no alterations of Shh and Su(Fu). This could be due to elevated expression of indian hedgehog, or even alterations of other components of the pathway (such as Rab23 or Fused).

Once hedgehog pathway is activated, the target gene expression will be up-regulated. Thus, analysis of target gene expression using immunohistochemistry will be an effective way to detect hedgehog pathway activation in prostate cancer. Currently, PTCH1, Gli1 and HIP are good markers for the hedgehog pathway.

Perspectives on prostate cancer therapy

Our findings not only provide novel basic understanding of prostate cancer, but also allow us to design new ways to treat prostate cancer. With a specific SMO antagonist, cyclopamine, it will be possible in the future to treat prostate cancers, which have over-expressed sonic hedgehog. However, as a downstream molecule, tumors with Su(Fu) inactivation may not respond to cyclopamine treatment. Therefore, additional small molecule inhibitors appear to be necessary to treat Su(Fu) inactivated prostate cancer. One possibility is to use Gli1 siRNA since we have indicated that down-regulation of Gli1 may be an important mechanism by which inhibition of the hedgehog pathway by cyclopamine induces apoptosis (Fig. 8). Sanchez et al also indicated that Gli1 siRNA down-regulated DNA synthesis in prostate cancer cells [24].

Conclusion

Taken together, our findings suggest that activation of the hedgehog pathway involves prostate cancer progression. There might be several mechanisms by which the hedgehog pathway is activated in advanced prostate cancers, including loss of Su(Fu) protein expression, over-expression of sonic hedgehog or other alterations. We demonstrate that activation of the hedgehog pathway is associated with DNA synthesis and cell invasiveness in prostate cancer cells. Inhibition of the hedgehog pathway, on the other hand, causes apoptosis possibly through down-regulation of Gli1. Our studies predict that targeted inhibition of the hedgehog pathway may be an effective way to prevent prostate cancer progression.

Materials and methods

Tissue Microarray of Prostate Cancer

A total of 55 paraffin-embedded tissue blocks from patients with prostate cancer were obtained from UTMB Surgical pathology with approval from the Institutional Review Board (IRB). Pathological reports, H&E staining of each specimen were reviewed to determine the nature of the disease and the Gleason scores. Of 55 specimens, 18 were from tumors with Gleason scores 3–6, 15 with Gleason score 7 and 22 with Gleason scores 8–10. The tumor area was first identified before tissue microarray (1.5 mm in diameter for specimens) was assembled with Beecher's Tissue arrayer-I[®] according to manufacturer's instruction <http://www.beecherinstruments.com>.

Immunohistochemistry and Western blotting

A standard avidin-biotin immunostaining technique was performed using a kit from Vector laboratories using specific antibodies to Su(Fu) (Santa Cruz Biotechnology Cat# 10933), PTCH1 (Santa Cruz Biotechnology Cat# 6149), HIP (R&D systems Cat# AF1568) and Shh (Santa Cruz Biotechnology Cat# 9024) and PSA (Vector laboratories). Positive staining was in red or brown. The specificity of antibodies was tested using the very peptide used for raising the antibodies, which abolished the specific staining. Hematoxylin was used for counterstaining (in blue). Protein was analyzed by Western analysis with appropriate antibodies [Su(Fu) antibodies were from Santa Cruz, beta-actin antibody was purchased from Sigma]. The signals were visualized with the enhanced chemiluminescence detection system (Amersham).

Cell lines and Cell invasion assay

Cell lines (RWPE-1, Du145, PC3, LN-CAP) were purchased from ATCC and cultured according to the suggested conditions. TSU was kindly provided by Dr. Allen Gao. Cell invasion assay was performed with BD Bio-coat cell invasion chambers according to manufacturer's instruction (BD Bioscience, Inc., Franklin Lakes, NJ), with triplicates for each sample and the experiment was repeated three times with the similar results. Cells were treated with 5 μ M cyclopamine (or tomatidine) before (for 12 h) and during cell invasion assay (for 24 h). The rate of cell invasion was calculated by dividing cell numbers penetrated the matrigels by the number of cells in the control chambers (without matrigels).

RT-PCR and sequencing analysis

Total RNA was isolated using Trizol[®] reagent (Invitrogen), and RT-PCR was performed using Promega's RT-PCR system according to the manufacturer's protocol. Two pairs of Su(Fu) primers were used (the first set with the forward primer 5'-cctacgcaccccgatggcg-3' and the reverse primer 5'-agccaaaaccactactctcca-3'; the second set with the forward primer 5'-tccaggttaccgctatcgtc-3' and the reverse primer 5'-tagttagcggactgtcg-3'). PCR products were first purified using Qiagen's Gel Extraction Kit. Due to existence of possible Su(Fu) splicing isoforms in humans, Su(Fu) genetic mutations were screened after the PCR products were cloned into TOPO[®] TA cloning vectors (Invitrogen). Several independent clones (from three experiments) of each PCR product were selected for sequencing analysis in UTMB sequencing facility. All mutations were confirmed by at least six independent clones.

Real-time PCR We used Applied Biosystems' assays-by-demand 20 $\times$ assay mix of primers and TaqMan probes (FAM[™] dye-labeled) for the target genes (human Gli and PTCH1, the sequences have been patented by Applied Biosystems, Foster City, CA) and pre-developed 18S rRNA

(VIC™-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) for an internal control. The primers are designed to span exon-exon junctions so as not to detect genomic DNA and the primers and probe sequences were searched against the Celera database to confirm specificity. To obtain the relative quantitation of gene expression, a validation experiment was performed to test the efficiency of the target amplification and the efficiency of the reference amplification. All absolute values of the slope of log input amount vs. ΔC_T were <0.1 . Separate tubes (singleplex) one-step RT-PCR was performed with 20 ng RNA for both target genes and endogenous control. The reagent we used was TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR was: reverse transcription 48°C for 30 min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 sec and annealing/extension 60°C for 1 min (repeat 40 times) on ABI7000. Triplicate C_T values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalization to an endogenous reference (18sRNA) and relative to a calibrator.

BrdU labeling and TUNEL assay

BrdU labeling was performed using an *in situ* cell proliferation kit (Roche Molecular Biochemicals) [22]. Cells were treated with $5\mu\text{M}$ cyclopamine (or tomatidine) for 12 h before BrdU labeling (1 h at 37°C). The percentage of BrdU positive cells was obtained by counting over 1000 cells under microscope, and the experiment was repeated twice with similar results. TUNEL assay was performed using an *in situ* cell death kit (Roche Molecular Biochemicals) [21,29]. Cells were treated with $5\mu\text{M}$ cyclopamine (or tomatidine) for 36 h before TUNEL assay).

List of abbreviations

PSA – prostate specific antigen; HIP – hedgehog-interacting protein; Su(Fu) – suppressor of fused; PTCH1 – human homologue of *patched* 1; Shh – sonic hedgehog; SMO – smoothened, BCC – basal cell carcinoma.

Authors' contributions

Tao Sheng contributed to Figures 6, 7, 8, cellular functions of the hedgehog pathway in prostate cancer cells. Chegxin Li contributed to primary tumor protein expression, particularly on Su(Fu) expression. Xiaoli Zhang contributed to mutation analyses of Su(Fu) in prostate cancer and real-time PCR analyses. Sumin Chi contributed to HIP antibody test (Fig. 3A and 3B). Nonggao He contributed to HIP antibody staining (Fig. 3C). Kai Chen contributed to PTCH1 antibody test (Fig. 1A). Frank McCormick involved in the initial project discussion. Zoran Gatalica

contributed to prostate cancer histology and Gleason scores of the tumors.

Additional material

Additional File 1

Table 1 Prostate cancer specimens and protein expression. Prostate cancer specimens and expression of several hedgehog signaling proteins are summarized in this table (A). A total of 55 specimens were used in this study. The Gleason scores and protein expression of Shh, PTCH1 and Su(Fu) are shown (B).

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Filed: October 9, 2003
For: USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL
CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit O

Xuan YH et al, *Modern Pathology* 2006;19:1139-1147.

Enhanced expression of hedgehog signaling molecules in squamous cell carcinoma of uterine cervix and its precursor lesions

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The hedgehog (Hh)-signaling pathway plays an essential role in normal development. Deregulation of this pathway is responsible for several types of cancers. The aim of this study was to determine the expression pattern and the extent of Hh-signaling molecules in squamous cell carcinoma of uterine cervix and its precursor lesions. A total of 106 uterine cervical cancers and related lesions (37 squamous cell carcinomas, 23 cervical intraepithelial neoplasia (CIN) III, 10 CIN II, four CIN I, 32 normal cervical epithelia) were immunohistochemically analyzed with anti-Shh, Indian Hh (Ihh), Patched (PTCH), Smoothened (Smo), Gli-1, Gli-2, Gli-3 antibodies on paraffin blocks. The results showed that the expression of all the Hh-signaling molecules was greatly enhanced in uterine cervical tumors, including carcinoma and its precursor lesions. The staining pattern was mainly cytoplasmic except for Gli-1/2, whose expression was observed in both cytoplasm and nucleus. In case of Ihh, PTCH, Smo and Gli-1, their expression in normal epithelium was completely absent or rare. The expression of all the seven Hh-signaling molecules mentioned above was significantly increased in CIN II/III and carcinoma, compared with that in normal epithelium ($P < 0.05$). The expression of Shh was increased by double; the first increase occurred in normal epithelium–CIN transition, and the second, during the progression of CIN to carcinoma. These results strongly suggest that the Hh-signaling pathways were extensively activated in carcinoma and CIN of uterine cervix. In conclusion, the Hh-signaling pathways may be involved in carcinogenesis of squamous cell carcinoma of uterine cervix and can be considered as a potential therapeutic target.

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Keywords: sonic hedgehog; patched; smoothened; Gli; squamous cell carcinoma; uterine cervix

Hedgehog (Hh) is a family of secreted proteins and the Hh-signaling pathway has been known to play central roles in directing the embryonic pattern formation during development and to be involved in the regulation of stem cell renewal in adult tissue.¹ In human development, the Hh-signaling pathway is

crucial for the decision making of left-right asymmetry and patterning of various organs including the brain, spinal cord, craniofacial structures, lung, teeth, eye, and hair. However, during the post-embryonic period, the Hh-signaling pathway has been known to be involved in the regulation of adult tissue stem cells during the regeneration of adult tissue after damage.^{1,2–4}

In mammals, three hedgehog (Hh) homologues have been identified: Sonic (Shh), Indian, and Desert. The Hh proteins activate a membrane-receptor complex and this, in turn, by means of cytoplasmic signal transduction, activates Gli zinc-

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finger transcription factors. The receptor complex is formed by Patched (PTCH) and Smoothened (Smo), where PTCH normally inhibits Smo. When Hh binds to PTCH, this repression of Smo by PTCH is released, allowing Smo to activate the Gli protein.⁵ The Gli proteins are large and multifunctional transcription factors, and there are three Gli proteins that behave differently with partially redundant functions; Gli1 and Gli2 can mediate Hh signals and have been implicated in tumorigenesis. Gli-1 is known to function primarily as an oncogene if tumors arise due to overexpression. On the other hand, Gli-2 and Gli-3 function as oncogenes or tumor suppressors, depending on the type of mutation and cellular context.⁶

The deregulation of Hh-signaling pathway has been implicated in several types of cancers.²⁻⁴ The mutational activation of the Hh-signaling pathway, whether sporadic or in Gorlin's syndrome, is associated with tumorigenesis in a small subset of these tissues, predominantly skin, the cerebellum, and skeletal muscle.^{7,8} Furthermore, extensive activation of the Shh-signaling pathways has been reported in cancers of other organs, such as small cell lung cancer,^{9,10} carcinomas of esophagus, stomach, pancreas, biliary tract, and prostate,¹ and colonic neoplasia.¹¹

Approximately 10 370 new cases of cervical cancers and 3710 deaths were anticipated in the United States in 2005.¹² Cervical cancer is also an important health problem in adult women in developing countries, where it is the most or second most common cancer among women.¹³ Cervical cancer claims the lives of 231 000 women annually worldwide.¹³ Even a conservative estimate of the global prevalence suggests that there are 1.4 million cases of clinically recognized cervical cancer each year. It is also estimated that 3-7 million women worldwide may have high-grade dysplasia.¹³

Although the importance of the Hh signaling in tumor development is recognized in various organs, there has been no study regarding the expression of Hh-signaling molecules in uterine cervical tumors.

In this study, we conducted a comprehensive analysis of the expression of Hh-signaling molecules at the protein level via immunohistochemistry in uterine cervical cancer and its precancerous lesions. The results demonstrated that the increased and persistent expression of Hh-signaling molecules may be implicated in carcinogenesis of uterine cervix.

Materials and methods

Patients, Tissue Samples, and Reagents

We investigated (106 cases of uterine cervical) carcinomas and their related lesions, obtained from the surgical pathology files at the Department of Pathology, Chungbuk National University Hospital. The criteria for inclusion were the histopathologic

diagnosis of uterine cervical lesions and the availability of paraffin-embedded tissue specimens. The selected cases consisted of 37 cases of squamous cell carcinoma, 23 cases of CIN III, 10 cases of CIN II, four cases of CIN I, and 32 cases of normal cervical (epithelium). This study was approved by the institutional review board of Chungbuk National University Hospital.

The pathologic slides were reviewed to analyze pathologic parameters, including tumor size, depth of invasion, and the presence of nodal metastasis. The 37 squamous cell carcinomas (age range = 22-69 years; average age = 46 years) encompassed 29 early cases (pTNM stage I = 27, pTNM stage II = 2) and eight advanced cases (pTNM stage III = 8). The TNM staging was assessed according to the staging system established by the American Joint Committee on Cancer (AJCC).¹⁴

Tissue microarray slides were employed for the purpose of effective detection. For preparation of these slides, we punched tissue columns (3.0 mm in diameter) from the original blocks and inserted them into new paraffin blocks (each containing 30 holes to accept the tissue columns). Consequently, serially sectioned slides were prepared. Each tissue microarray slide (1 × 3 in) could hold 30 specimens, allowing us to analyze 30 specimens simultaneously with a minimum variation during the staining process. Each specimen was round in shape and 3.0 mm in diameter, thereby providing a sufficient amount of tissue for histopathologic analysis.

All archival materials were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (4 μm) were prepared on silane-coated slides (Sigma, St Louis, MO, USA). The immunostaining kits were purchased from DAKO Inc. (Glostrup, Denmark).

The Immunohistochemical Staining Procedure

The tissue sections in the microslides were deparaffinized with xylene, hydrated in serial dilutions of alcohol, and immersed in 3% H₂O₂ to quench endogenous peroxidase activity. The sections were then microwaved in 40 mM Borate buffer (pH 8.2) supplemented with 1 mM EDTA and 1 mM NaCl for 20 min for antigen retrieval.^{15,16} Tris-EDTA buffer (Tris 40 mM, EDTA 1 mM, pH 10.0) was also used as a retrieval buffer. The tissues were then incubated with several primary antibodies (anti-Shh, anti-Ihh, anti-PTCH, anti-Smo, anti-Gli1, anti-Gli2, and anti-Gli3). The dilution ratio and optimal retrieval buffer of each antibody are shown in Table 1. Primary antibody incubation was carried out for 60 min, followed by three successive rinsings with a washing buffer. Further incubation was performed with dextran polymer conjugated with peroxidase and goat anti-rabbit Ab (DAKO, Envision plus) for an additional 20 min at room temperature. After rinsing, the slides were washed and the chromogen

Table 1 The antibodies and retrieval buffers for each molecule

	Clonality	Source	Catalogue number	Dilution ratio	Retrieval buffer
Shh	Rabbit polyclonal	Santa-cruz	sc-9024	1:30	Borate buffer
Ihh	Rabbit polyclonal	Santa-cruz	sc-13088	1:25	Borate buffer
PTCH	Rabbit polyclonal	Santa-cruz	sc-9016	1:25	Tris-EDTA buffer
Smo	Rabbit polyclonal	Santa-cruz	sc-13943	1:50	Borate buffer
Gli-1	Rabbit polyclonal	Santa-cruz	sc-20687	1:50	Tris-EDTA buffer
Gli-2	Rabbit polyclonal	Abcam Ltd	ab-7181	1:100	Borate buffer
Gli-3	Rabbit polyclonal	Santa-cruz	ac-20688	1:40	Borate buffer

was developed for 5 min with liquid 3, 3'-diaminobenzidine (DiNonA, Seoul, South Korea). The slides were counterstained with Meyer's hematoxylin, dehydrated, and mounted with Canada balsam for examination. We used distilled water with 0.1% tween 20 as a rinsing solution.¹⁷

Evaluation of Results of Immunohistochemical Staining

We used the Sinicrope *et al*'s¹⁸ scoring method (1995) to evaluate both the intensity of immunohistochemical staining and the proportion of the stained epithelial cells. The staining intensity was subclassified as follows: 1, weak; 2, moderate; or 3, strong. The positive cells were quantified as a percentage of the total number of epithelial cells and the proportions were assigned to one of five categories: 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. The percentage of positivity of the tumor cells and the staining intensity were then multiplied in order to generate the immunoreactivity score (IS) for each of the tumor specimens. Cytosolic and nuclear stainings were independently analyzed. Each lesion was separately examined and scored by two pathologists (XYH and SHK). Cases with discrepant scores were discussed to obtain a consensus.

Statistical Analysis

Statistical analyses were conducted using Fisher's exact tests, Pearson's χ^2 tests, ANOVA, Mann-Whitney tests, Kruskal-Wallis test, Tukey's HSD, and Duncan's test (as a *post hoc* test). *P*-values <0.05 were regarded to be statistically significant. All statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA).

Results

The Expression Pattern of Hh-Signaling Molecules in Squamous Cell Carcinoma of Uterine Cervix and Its Precursor Lesions

We analyzed the expression pattern of Hh-signaling molecules (ie Shh, Indian Hh (Ihh), PTCH, Smo, Gli-

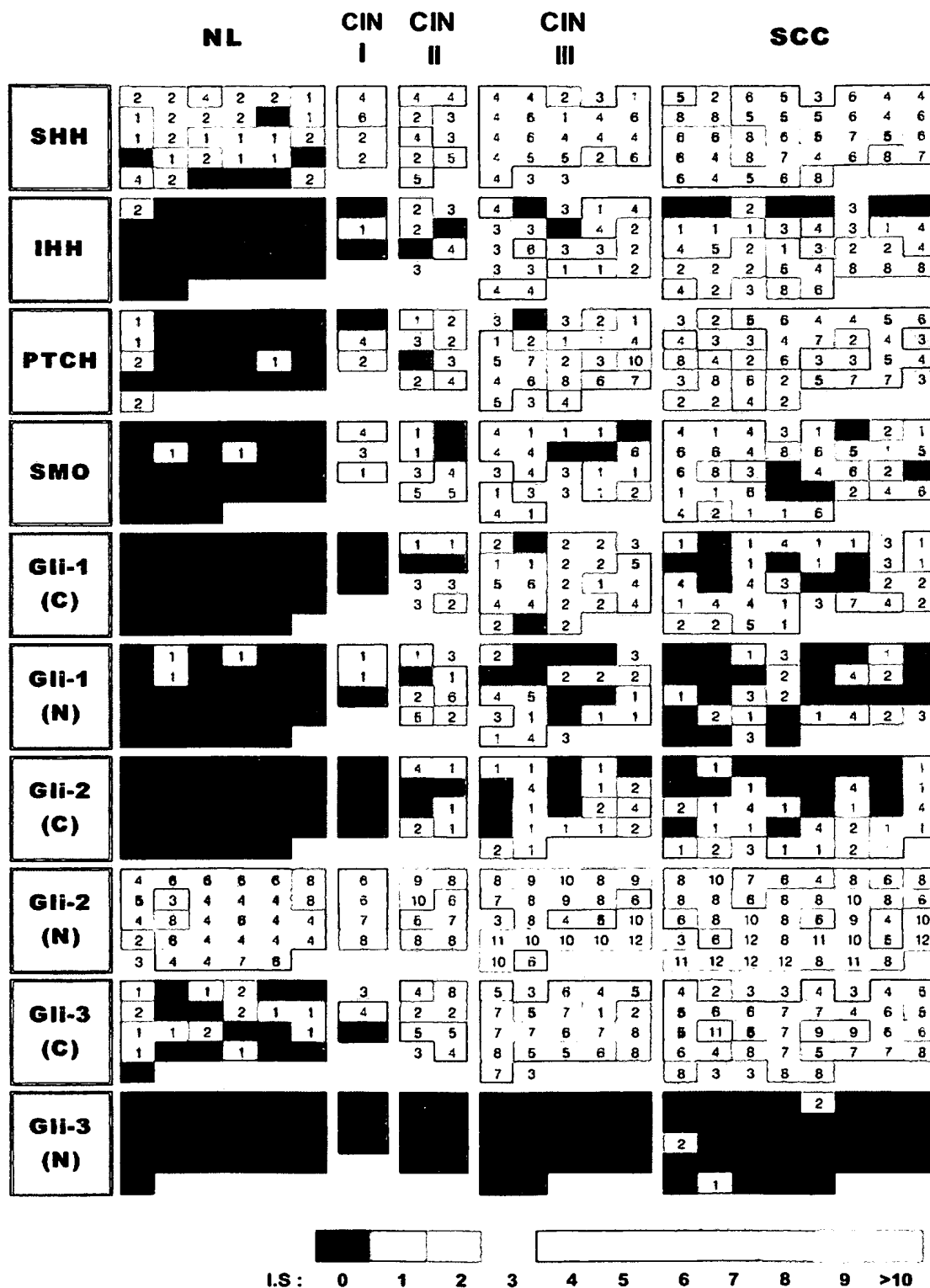
1, Gli-2, Gli-3) in squamous cell carcinoma and its precursor lesions of uterine cervix via immunohistochemistry. First, the average intensity was analyzed (the mean of IS from immunostaining). Then the Shh expression in terms of the rate of high expression was analyzed.

Shh expression

In case of Shh, the expression was observed in the cytoplasm of both glandular component and squamous epithelium of normal uterine cervical epithelium; however, the level was low (IS: 1.5 ± 1.1). In squamous epithelium of the cervix, Shh was mainly expressed in basally located cells (Figures 1 and 2). However, its expression level was significantly increased in cervical intraepithelial neoplasia (CIN I, IS: 3.5 ± 1.9 ; CIN II, IS: 3.4 ± 1.2 ; CIN III, IS: 3.8 ± 1.5) compared with in normal epithelium (IS: 1.5 ± 1.1) ($P < 0.005$) (Figures 1 and 3, Table 2). The level of Shh expression was also increased significantly in squamous cell carcinoma (IS: 5.7 ± 1.5) compared with in CINs with a statistical significance ($P < 0.001$) (Figures 1 and 3, Table 2). We also analyzed the Shh expression in terms of the rate of high expression, which was defined by IS higher than 3 (Table 3). In normal epithelium Shh high expression was rare (3/32, 9%). The rate was significantly increased in CIN (CIN I: 2/4, 50%. CIN II: 6/10, 60%. CIN III: 15/22, 68%) and even higher in carcinomas (35/37, 95%) ($P < 0.001$) (Table 3).

Ihh expression

The Ihh expression was very rare and almost completely absent in normal uterine cervical epithelium. However, aberrant expression of Ihh was frequently observed in CIN II, CIN III and squamous cell carcinoma (Figures 1 and 3). The level of Ihh expression was significantly increased in CIN II (IS: 2.0 ± 1.5), CIN III (IS: 2.7 ± 1.5) and carcinoma (IS: 2.9 ± 2.4), compared with in normal epithelium (IS: 0.1 ± 0.4) ($P < 0.001$) (Table 2). The rate of high expression (IS > 3) was zero in normal epithelium (0/25) and was significantly increased in CIN III (6/22, 27%) and carcinoma (13/37, 35%) ($P < 0.05$) (Table 3).



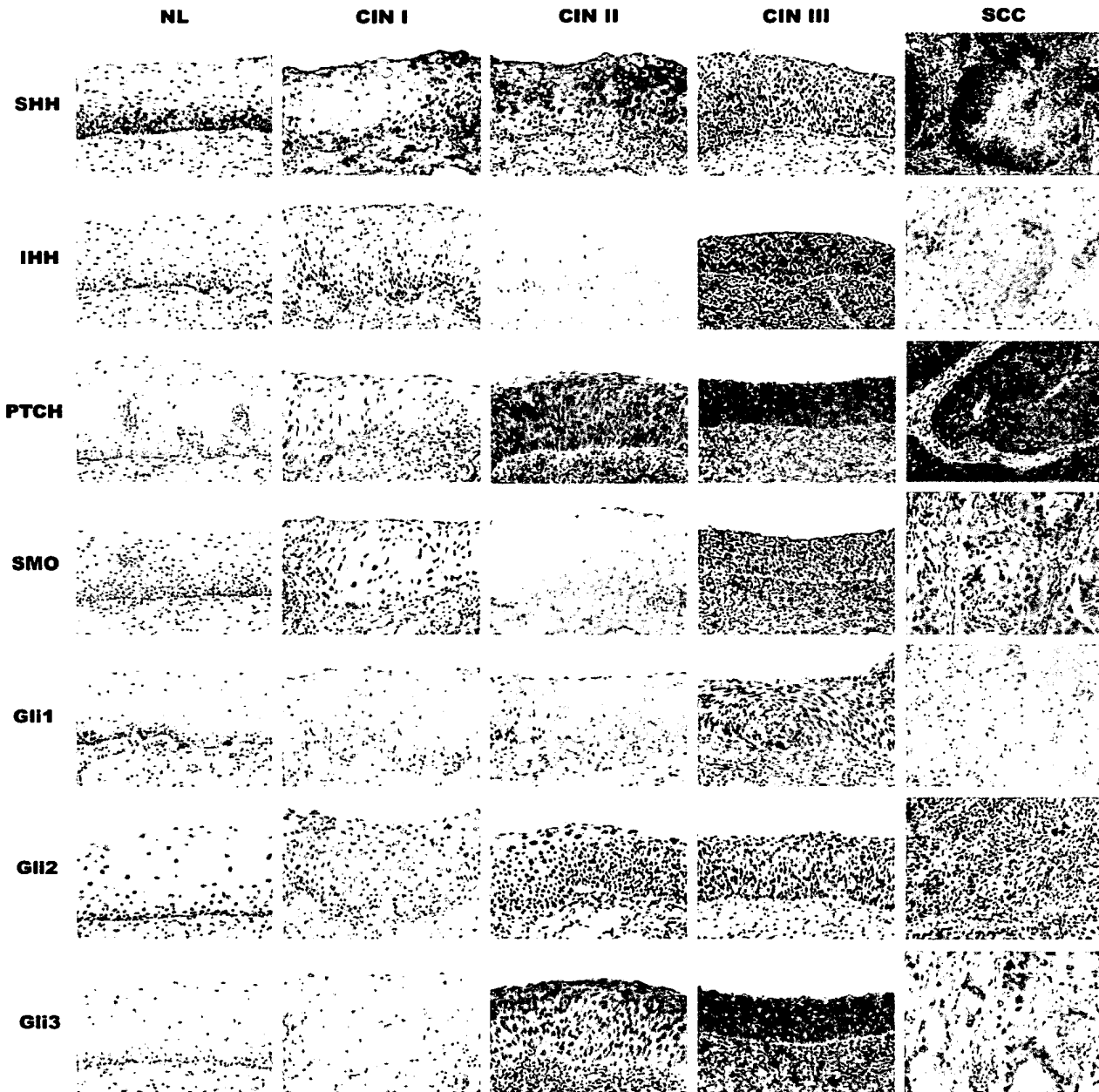


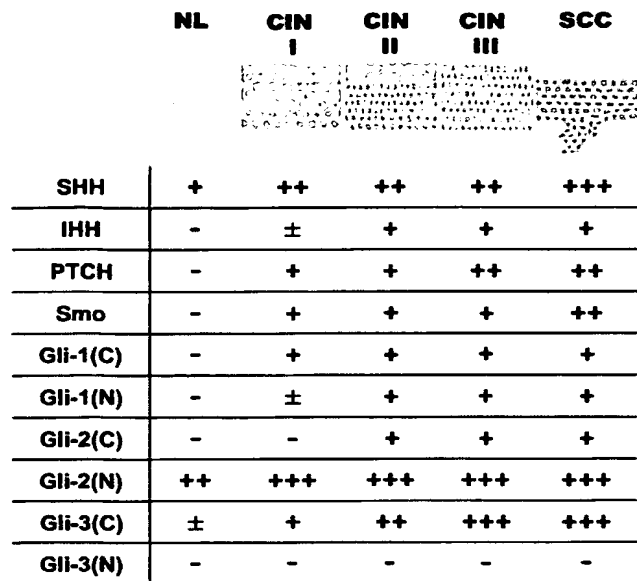
Figure 2 The immunostaining of Hh-signaling molecules in the uterine cervical lesions including carcinoma and its precursor lesions; NL, normal uterine cervical epithelium; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.

PTCH expression

In normal epithelium *PTCH* was expressed in 5 of 25 cases (20%) (Figure 1). The average staining

intensity (IS) increased significantly in CIN II (IS: 2.0 ± 1.5), CIN III (IS: 2.7 ± 1.5), and carcinoma (IS: 2.9 ± 2.4), compared with normal epithelium

Figure 1 A schematic representation of the expression of Hh-signaling molecules in squamous cell carcinoma of uterine cervix and its related lesions; NL, normal uterine cervical epithelium; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; Gli-1(C), cytoplasmic staining of Gli-1; Gli-1(N), nuclear staining of Gli-1; Gli-2(C), cytoplasmic staining of Gli-2; Gli-2(N), nuclear staining of Gli-2; Gli-3(C), cytoplasmic staining of Gli-3; Gli-3(N), nuclear staining of Gli-3. The expression level of individual cases of each disease entity represented by immunoreactivity score (IS) is shown in colors.



	NL	CIN I	CIN II	CIN III	SCC
SHH	+	++	++	++	+++
IHH	-	±	+	+	+
PTCH	-	+	+	++	++
Smo	-	+	+	+	++
Gli-1(C)	-	+	+	+	+
Gli-1(N)	-	±	+	+	+
Gli-2(C)	-	-	+	+	+
Gli-2(N)	++	+++	+++	+++	+++
Gli-3(C)	±	+	++	+++	+++
Gli-3(N)	-	-	-	-	-

Figure 3 A schematic representation and the summary of the results from this study.

(IS: 0.1 ± 0.4) ($P < 0.001$) (Table 2). The rate of high expression (IS > 3) was low in normal epithelium (0/25, 0%) and CIN II (1/9, 11%), but substantially and significantly increased in CIN III (11/22, 50%) and carcinoma (22/37, 60%) with a statistical significance ($P < 0.001$) (Table 3).

Smo expression

Smo expression was also rare and was found in only two out of 27 cases (Figure 1). The average intensity was extremely low in normal epithelium (IS: 0.1 ± 0.3) and was significantly increased in neoplastic lesions including CIN I/II/III (IS: $2.7 \pm 1.5/2.6 \pm 2.1/2.1 \pm 1.7$, respectively), and carcinoma (IS: 3.2 ± 2.4) ($P < 0.001$) (Figures 1 and 3, Table 2). The rate of high expression (IS > 3) was also zero in normal epithelium (0/27, 0%), but was increased significantly in tumorous lesions such as CIN I (1/3, 33%)/CIN II (4/9, 44%)/CIN III (5/21, 24%) and carcinoma (18/37, 49%) ($P < 0.001$) (Table 3).

Gli-1 expression

In contrast to the other Shh-signaling molecules whose expressions were observed mainly in cyto-

Table 2 The mean of immunostaining score of Hh-signaling molecules in squamous cell carcinoma of uterine cervix and its precursor lesions

Diagnosis	No.	Shh	Ihh	PTCH	Smo	Gli-1 (C)	Gli-1 (N)	Gli-2 (C)	Gli-2 (N)	Gli-3 (C)
Carcinoma	37	5.7 ± 1.5	2.9 ± 2.4	4.2 ± 1.8	3.2 ± 2.4	1.9 ± 1.7	1.0 ± 1.3	1.1 ± 1.3	8.2 ± 2.4	5.8 ± 2.1
CIN III	23	3.8 ± 1.5	2.7 ± 1.5	3.9 ± 2.6	2.1 ± 1.7	2.6 ± 1.6	1.4 ± 1.5	1.1 ± 1.2	8.2 ± 2.3	5.5 ± 2.0
CIN II	10	3.4 ± 1.2	2.0 ± 1.5	2.2 ± 1.2	2.6 ± 2.1	1.6 ± 1.3	2.3 ± 2.3	1.1 ± 1.4	7.7 ± 1.4	4.1 ± 2.0
CIN I	4	3.5 ± 2.0	1.0 ± 1.0	2.0 ± 2.0	2.7 ± 1.5	0.0 ± 0.0	0.7 ± 0.6	0.0 ± 0.0	6.7 ± 1.0	2.3 ± 2.1
Normal epithelium	32	1.5 ± 1.1	0.1 ± 0.4	0.3 ± 0.6	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	4.9 ± 1.6	0.8 ± 0.9
P-value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

IS, immunostaining score; CIN, cervical intraepithelial neoplasm; NL, normal epithelium.

Shh: NL vs CIN I/II/III and carcinoma: $P < 0.025$, CIN I/II/III vs carcinoma: $P < 0.05$.

PTCH: NL vs CIN II/III and carcinoma: $P < 0.005$, CIN II vs carcinoma: $P < 0.005$.

Smo: NL vs CIN I/II/III and carcinoma: $P < 0.001$, Ihh: NL vs CIN II/III and carcinoma: $P < 0.005$.

Gli1(C): NL vs CIN II/III and carcinoma: $P < 0.001$, CIN I vs CIN III and carcinoma: $P < 0.025$.

Gli1(N): NL vs CIN II/III and carcinoma: $P < 0.01$.

Gli2(C): NL vs CIN II/III and carcinoma: $P < 0.01$, Gli2(N): NL vs CIN I/II/III and carcinoma: $P < 0.05$.

Gli3(C): NL vs CIN II/III and carcinoma: $P < 0.001$, CIN I/II vs carcinoma: $P < 0.05$.

Table 3 The percentage of high expression of Hh-signaling molecules in the squamous cell carcinoma of uterine cervix and its precursor lesions

Diagnosis	No.	Shh	Ihh	PTCH	Smo	Gli-1 (C)	Gli-1 (N)	Gli-2 (C)	Gli-2 (N)	Gli-3 (C)
Carcinoma	37	35/37 (95%)	13/37 (35%)	22/37 (60%)	18/37 (49%)	8/36 (22%)	2/36 (6%)	4/38 (11%)	26/38 (68%)	30/36 (83%)
CIN III	23	15/22 (68%)	6/22 (27%)	11/22 (50%)	5/21 (24%)	7/23 (30%)	3/23 (13%)	2/22 (9%)	16/22 (73%)	18/22 (82%)
CIN II	10	6/10 (60%)	1/7 (14%)	1/9 (11%)	4/9 (44%)	0/8 (0%)	2/8 (25%)	1/8 (13%)	5/8 (63%)	5/8 (63%)
CIN I	4	2/4 (50%)	0/3 (0%)	1/3 (33%)	1/3 (33%)	0/3 (0%)	0/3 (0%)	0/4 (0%)	1/4 (25%)	1/3 (33%)
Normal epithelium	32	3/32 (9%)	0/25 (0%)	0/25 (0%)	0/27 (0%)	0/29 (0%)	0/29 (0%)	0/29 (0%)	3/29 (10%)	0/25 (0%)
P-value		< 0.001	< 0.05	< 0.001	< 0.001	< 0.025	0.102	0.471	< 0.001	< 0.001

CIN, cervical intraepithelial neoplasm. NL, normal epithelium.

Shh: NL vs CIN I/II/III and carcinoma: $P < 0.05$, CIN I/II/III vs carcinoma: $P < 0.01$.

PTCH: NL vs CIN I/III and carcinoma: $P < 0.01$, CIN II vs CIN III and carcinoma: $P < 0.05$.

Smo: NL vs CIN I/II/III and carcinoma: $P < 0.01$, Ihh: NL vs CIN III and carcinoma: $P < 0.01$.

Gli1(C): NL vs CIN III and carcinoma: $P < 0.01$.

Gli2(N): NL vs CIN II/III and carcinoma: $P < 0.01$.

Gli3(C): NL vs CIN I/II/III and carcinoma: $P < 0.01$, CIN I vs carcinoma: $P < 0.05$.

plasm, Gli-1/2 molecules were expressed in both cytoplasm and nucleus. The cytoplasmic expression of Gli-1 was completely absent in normal epithelium, but aberrant expression of the protein was frequently observed in CIN II/III and carcinoma (Figure 1). The average intensity of cytoplasmic Gli-1 expression significantly increased in CIN II (IS: 1.6 ± 1.3)/CIN III (IS: 2.6 ± 1.6) and carcinoma (IS: 1.9 ± 1.7), compared with in normal cervical epithelium (IS: 0.0 ± 0.0) ($P < 0.001$) (Table 2). The rate of high cytoplasmic expression (IS > 3) was zero in normal epithelium (0/29) and CIN I/II (0/3 and 0/8), but significantly increased in CIN III (7/23, 30%) and carcinoma (8/36, 22%) ($P < 0.01$) (Table 3).

The nuclear expression of Gli-1 was very weak and rare in normal cervical epithelium (Figures 1 and 2). The average intensity of nuclear expression significantly increased in CIN II (IS: 2.3 ± 2.3)/CIN III (IS: 1.4 ± 1.5) and carcinoma (IS: 1.0 ± 1.3), compared with in normal epithelium (IS: 0.1 ± 0.3) ($P < 0.001$) (Table 2). The rate of high nuclear expression (IS > 3) was generally low in overall cervical lesions without a significant difference (Table 3).

Gli-2 expression

The cytoplasmic expression of Gli-2 was completely absent in normal epithelium, but aberrant expression of the protein was frequently noted in CIN II/III and carcinoma in the same manner as in Gli-1 (Figures 1 and 2). The average intensity of cytoplasmic Gli-2 expression significantly increased in CIN II (IS: 1.1 ± 1.4)/CIN III (IS: 1.1 ± 1.2) and carcinoma (IS: 1.1 ± 1.3), compared with in normal epithelium (IS: 0.0 ± 0.0) ($P < 0.001$) (Table 2). The rate of high cytoplasmic expression (IS > 3) was generally low in overall cervical lesions without a significant difference (Table 3).

The nuclear expression of Gli-2 was very abundant in normal epithelium in contrast to cytoplasmic staining (Figure 1). Nuclear staining was also found in endocervical glands as well as squamous epithelium. The average intensity of nuclear expression significantly increased in CIN I (IS: 6.8 ± 1.0)/CIN II (IS: 7.7 ± 1.4)/CIN III (IS: 8.2 ± 2.3) and carcinoma (IS: 8.2 ± 2.4) ($P < 0.001$), compared with in normal epithelium (IS: 4.9 ± 1.6) (Table 2). The rate of high nuclear expression (IS > 7) was low in normal epithelium (3/29, 10%), but significantly increased in CIN II/III (5/8, 63% and 16/22, 73% respectively) and carcinoma (26/38, 68%) ($P < 0.001$) (Table 3).

Gli-3 expression

In contrast to Gli-1/2, the majority of Gli-3 expression was cytoplasmic. The nuclear expression was observed in only three cases out of 36 carcinomas. There was no nuclear expression in the normal epithelium and CIN I/II/III lesions (Figure 1). Conversely, the cytoplasmic expression of Gli-3 was relatively abundant in normal epithelium. Cytoplasmic staining was also focally found in

endocervical glands as well as in squamous epithelium. The average intensity of cytoplasmic expression was significantly increased in CIN II (IS: 4.1 ± 2.0)/CIN III (IS: 5.6 ± 2.0) and carcinoma (IS: 5.8 ± 2.1), compared with normal epithelium (IS: 0.8 ± 0.9) ($P < 0.001$) (Table 2). And the average intensity of Gli-3 cytoplasmic expression in carcinoma was significantly higher than in CIN I/II ($P < 0.05$). On the other hand, the rate of high cytoplasmic expression (IS > 3) was zero in normal epithelium (0/25, 0%), but significantly increased in CIN II/III (5/8, 63% and 18/22, 82%, respectively) and carcinoma (30/36, 83%) ($P < 0.001$) (Table 3).

Discussion

In this study, we characterized the expression pattern of Hh-signaling molecules via immunohistochemistry in squamous cell carcinoma of uterine cervix and its precursor lesions. Our results are summarized in Table 3 and indicate that the expression of Hh-signaling molecules is greatly enhanced over the whole normal epithelium-CIN I/II/III-carcinoma sequence in the uterine cervix. To the best of our knowledge, this is the first study of expression of Hh-signaling molecules in carcinoma and CIN of uterine cervix. The expression of all Hh-signaling molecules is upregulated in neoplastic lesions including CIN and carcinoma, compared with normal epithelium. The expression of Ihh, PTCH, Smo, and Gli-1 was completely absent or rare in normal epithelium, whereas frequent and aberrant expression of these molecules was observed in neoplastic lesions. These results strongly suggest that the Hh-signaling pathway was extensively activated in carcinoma and CIN of uterine cervix.

There has been no study regarding the expression of Hh-signaling molecules in uterine cervix. The significance and functional implication of the Hh-signaling pathway in normal uterine cervical epithelium are unknown. Based on our results, it is postulated that the Hh-Gli-signaling pathway is not functioning in normal cervical epithelium despite the prevalent expression of Shh and Gli-2,3 because of absence or very rare expression of PTCH and Smo, which are major mediators in the Hh signaling. Instead, it is possible that Shh may affect other uncharacterized signaling pathways rather than the Shh-Gli pathway, and Gli-2 may also be regulated by other signaling pathways such as fibroblast growth factor (FGF) signaling² in normal cervix. The functional significance of Gli-3 as a transcription factor is questionable because its expression was relatively restricted to the cytoplasm instead of nucleus.

In the neoplastic lesions, the expression of PTCH and Smo were dramatically increased. Thus, it can be postulated that the Hh-Gli pathway is established. The significance of the Hh-signaling pathway in neoplastic lesions of uterine cervix is still elusive.

Generally, the Hh-signaling pathways are known to function postembryonically in stem cell renewal, tissue repair, and regeneration. When aberrantly and persistently activated by chronic tissue injury, this pathway may play an important role in the initiation and growth of cancer.¹ Hence, the Hh-signaling pathway as well as WNT signaling can be considered as a potential link between chronic tissue injury and cancer. The first link between Hh signaling and tumor formation was noted in patients with a familial cancer syndrome, basal cell-nevus syndrome (Gorlin's syndrome) that is associated with a PTCH mutation.² Familial PTCH mutations that activate the Hh pathway have been associated with an increased incidence of cancers in brain, skin, skeletal muscle in humans and mice. Additional studies in which the Hh pathway activities were antagonized by drugs such as cyclopamine, antibodies, and overexpression of negatively acting pathway components demonstrated an ongoing requirement for the Hh pathway activity in small cell lung cancer and carcinomas of esophagus, stomach, pancreas, colon, biliary tract, and prostate.¹ In addition, a consistent expression of Gli2 and Gli3 was observed in breast carcinomas.² It is intriguing that the Hh pathway activation in tissues that gives rise to non-Gorlin's tumors seems to be limited not by the ligand availability but by the responsiveness to the ligand. In normal prostate, the limiting factor for ligand responsiveness is Smo, which is not expressed in normal prostate tissue, and high-level activation of Hh pathway mediators including Smo occurs only in cancer cells. Collectively, these results are highly consistent with our findings.¹

An important factor in the carcinogenesis of uterine cervix is HPV infection, especially in high-risk groups. E6 proteins derived from high-risk HPV (type 16, 18, or 31) inactivate p53 by enhancing its degradation through ubiquitin-dependent proteolysis.¹⁹ It is intriguing that the hyperactivation of Hh-Gli signaling synergizes with loss of other tumor suppressor genes, especially p53. In other words, tumorigenesis in the *Ptc*^{+/-} mice was greatly enhanced in a p53-null background.²⁰ Therefore, exaggerated and inappropriate activation of the Hh-signaling pathway and inactivation of p53 by E6 proteins from the HPV may exert a synergistic effect on the carcinogenesis of uterine cervix.

Previously we observed a two-fold increase of Shh in gastric cancer.²¹ Shh expression is increased two-fold over the whole metaplasia-dysplasia early and advanced gastric carcinoma. The first increase was observed in metaplasia-dysplasia (adenoma) transition. The second occurred during the progression of early gastric cancer to advanced gastric cancer. This expression pattern is very similar to that observed in cervical neoplasia. The implications of these findings remain to be clarified. However, these results indicate that the role of Shh may change in a dosage-dependent manner and acquire additional tumor-promoting functions at higher expression.

(LOH at the PTCH locus was detected in 15.6% of squamous cell carcinoma cases (5/32) of uterine cervix;²² LOH, as well as an elevation of mRNA level of PTCH was found in basal cell carcinoma (BCC). This indicates that constitutive activation of Shh-PTCH signaling is required for the development of BCC.²³ It was reported that *PTCH*^{+/-} mice have a higher incidence of squamous cell carcinomas after ultraviolet (UV) exposure, and the size of the tumor is also greatly increased.²⁴ Therefore, it is possible to postulate that genetic alteration or deregulation of the expression of PTCH superimposed with another genetic alteration such as UV exposure or p53 alteration by HPV may play a role in the development of squamous cell carcinoma of uterine cervix.

Gli-1 is known to function primarily as an oncogene if tumors arise due to overexpression. However, Gli-2 and Gli-3 could function either as oncogenes or tumor suppressors, depending on the type of mutation and cellular context.⁶ However, Gli-2 is known to have a redundant function with Gli-1 and has been implicated in tumorigenesis.² According to our results, Gli-2 may play a more important role than Gli-1. The Gli-1 expression in the nucleus is relatively frequent but is negative in a considerable portion of tumor cases, whereas nuclear expression of Gli-2 is strong and persistent in all tumor cases. The nuclear expression of Gli-3 is very rare in tumor cases. Thus, the role of Gli-3 as a transcription factor is considered minimal.

In summary, we demonstrated that the expression of Hh-signaling molecules is greatly enhanced in uterine cervical tumors including carcinoma and its precursor lesions. Although the functional significance of the Hh pathway remains to be determined in uterine cervical cancer, the Hh-signaling pathway may play an important role in tumorigenesis and could be a potential therapeutic target.

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CELL CARCINOMA AND OTHER TUMORS
Group Art Unit: 1614

DECLARATION OF DR. SINAN TAS

Exhibit P

Kumamoto H et al, *Journal of Oral Pathology and Medicine* 2004;33:185-190.

Expression of Sonic hedgehog (SHH) signaling molecules in ameloblastomas

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BACKGROUND: To clarify the roles of Sonic hedgehog (SHH) signal transduction in oncogenesis and cytodifferentiation of odontogenic tumors, expression of SHH, Patched (PTC), Smoothened (SMO), and GLII was analyzed in ameloblastomas as well as in tooth germs.

METHODS: Tissue specimens of 9 tooth germs, 36 benign ameloblastomas, and 1 malignant ameloblastoma were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry for the expression of SHH, PTC, SMO, and GLII.

RESULTS: Expression of SHH, PTC, SMO, and GLII mRNA was detected in all tooth germ and ameloblastoma samples. Immunohistochemical reactivity for SHH, PTC, SMO, and GLII was detected in both normal and neoplastic odontogenic tissues. Expression of SHH, PTC, and GLII was more evident in epithelial cells than in mesenchymal cells, whereas SMO reactivity was marked in both epithelial and mesenchymal components in tooth germs and ameloblastomas. In ameloblastomas, these SHH signaling molecules were expressed more intensely in peripheral columnar or cuboidal cells than in central polyhedral cells; keratinizing cells and granular cells showed no or little reactivity.

CONCLUSION: Expression of SHH, PTC, SMO, and GLII in tooth germs and ameloblastomas suggests that these SHH signaling molecules might play a role in epithelial–mesenchymal interactions and cell proliferation in tooth development as well as in growth of these epithelial odontogenic tumors.

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Keywords: ameloblastoma; GLII; Patched (PTC); Smoothened (SMO); Sonic hedgehog (SHH)

Introduction

Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histologic variation and are classified into several benign and malignant entities (1–4). Ameloblastoma is the most frequently encountered tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with a high risk of recurrence (1, 2, 4). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types (1). Malignant ameloblastoma is defined as a neoplasm in which the pattern of an ameloblastoma and cytologic features of malignancy are shown by the primary growth in the jaws and/or by any metastatic growth (1). Recently, malignant ameloblastoma has been subclassified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytologic malignant features (3). Several recent studies have detected genetic and cytogenetic alterations in these epithelial odontogenic tumors (5–8); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

Sonic hedgehog (SHH) is a mammalian homolog of *Drosophila* segment polarity gene, *hedgehog (Hh)*, and encodes a secreted protein that activates a membrane-receptor complex formed by Patched (PTC) and Smoothened (SMO) (9, 10). PTC is a membrane-bound protein with 12 transmembrane domains (11), and SMO is another membrane-bound protein with seven transmembrane domains and characteristics of G protein-coupled receptors (12). PTC inhibits SMO in the absence of SHH, whereas binding of SHH to PTC suspends this inhibition, thereby activating a transcriptional factor, GLII (10). A zinc finger DNA-binding protein, GLII, mediates SHH signals from cytoplasm to nucleus (10, 13). SHH signal transduction plays a central role in the patterning of the limb, axial skeleton, central nervous system, lungs, digestive tract, dermal appendages, and teeth (14–17). Inherited or sporadic alterations in SHH-signaling pathway genes have been implicated in a number of developmental defects, and aberrant activation of SHH-signaling pathway during adult life has resulted in tumor formation (18–25).

Our previous studies confirmed cellular kinetics, including proliferation and cell death modulators, in tooth germs

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Table 1 Primers and antibodies

	Primer			Antibody		
	Sequence (5'-3')	Anneal (°C)	Product (bp)	Clonality	Source	Dilution
SHH	Forward: GAAGATCTCCAGAACTCC Reverse: TCGTAGTGCAGAGACTCC	57	233	Polyclonal (Goat IgG)	Santa Cruz Biotechnology Santa Cruz, CA, USA	1:100
PTC	Forward: TCCCAAGCAAATGTACGAGCA Reverse: TGAGTGGAGTTCTGTGCGACAC	55	144	Polyclonal (Goat IgG)	Santa Cruz Biotechnology	1:100
SMO	Forward: CTGGTACGAGGACGTGGAGG Reverse: AGGGTGAAGAGCGTGCAGAG	55	140	Polyclonal (Goat IgG)	Santa Cruz Biotechnology	1:80
GLI1	Forward: CAGAGAATGGAGCATCCTCC Reverse: TTCTGGCTCTTCCTGTAGCC	55	412	Polyclonal (Goat IgG)	Santa Cruz Biotechnology	1:100
GAPDH	Forward: GGAGTCAACGGATTGGT Reverse: GTGATGGGATTTCATTGAT	55	206			

and ameloblastomas, suggesting that these factors are associated with oncogenesis or cytodifferentiation of odontogenic epithelium (26–31). Alterations of *PTC* have been studied in odontogenic keratocysts (32, 33); however, investigations of SHH signaling molecules in odontogenic tumors are scant (5, 34). In the present study, expression of SHH, *PTC*, *SMO*, and *GLI1* was examined in ameloblastomas as well as in tooth germs using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry to clarify the possible role of SHH signal transduction in epithelial odontogenic tumors.

Materials and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry.

Tissue preparation

Specimens were surgically removed from 37 patients with ameloblastoma at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital. The tumors were divided into several parts. The first part was fixed in 4% paraformaldehyde phosphate buffer for one to several days and was embedded in paraffin. The tissue blocks were sliced and stained with hematoxylin and eosin for histologic diagnosis according to the WHO histologic typing of odontogenic tumors (4). The tumors comprised 36 ameloblastomas and 1 malignant ameloblastoma. The ameloblastomas were divided into 24 follicular and 12 plexiform types, including 12 acanthomatous and 2 granular cell subtypes. The malignant ameloblastoma was compatible with a metastasizing ameloblastoma according to the criteria provided by Eversole (3). The second part of each tumor was immediately frozen on dry ice and stored at -80°C until RT-PCR analysis. The third part of each tumor was embedded in Tissue-Tek OCT Compound (Sakura Finetechnical, Tokyo, Japan), quick-frozen in a mixture of acetone and dry ice, and stored at -80°C until immunohistochemical examination. Tooth germs of the mandibular third molars, enucleated from nine patients for orthodontic reasons at the stage of crown mineralization, were similarly prepared and compared with the ameloblastomas.

RT-PCR

Total RNA was extracted from each frozen tissue using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to

the manufacturer's protocol. First-stranded complementary DNA (cDNA) was synthesized from 1 μg of RNA using an Omniscript RT Kit (Qiagen) with oligo-(dT)₁₅ primer (Roche Diagnostics, Mannheim, Germany) as outlined by the manufacturer. The cDNA samples were amplified using a HotstarTaq Master Mix Kit (Qiagen) with specific primers in a DNA thermal cycler (Eppendorf, Hamburg, Germany). Primers used for detection of SHH, *PTC*, *SMO*, and *GLI1* are listed in Table 1. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), was used as an internal control for the examination of human gene expression. PCR was performed in a total volume of 50 μl , containing 0.5 μg of template cDNA and 0.5 μM of each specific primer set. The procedure for amplification included 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C (for SHH) or 55°C (for *PTC*, *SMO*, *GLI1*, and *GAPDH*) for 45 s and elongation at 72°C for 60 s with heat starting at

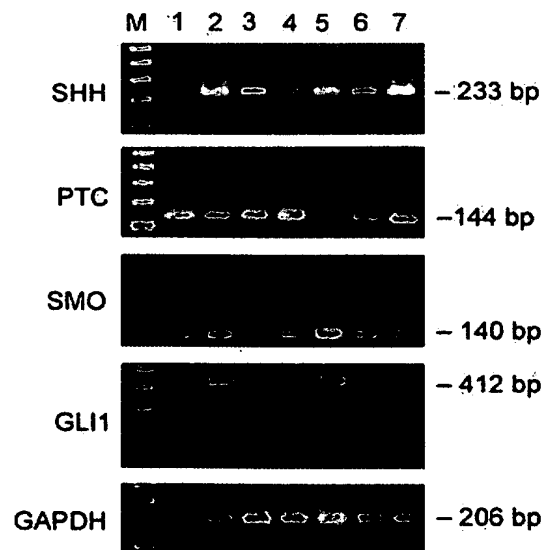


Figure 1 RT-PCR analysis of SHH, *PTC*, *SMO*, and *GLI1* mRNA expression in tooth germs and ameloblastomas (M: molecular-weight standard; 1, 2: tooth germs; 3–6: ameloblastomas; and 7: malignant ameloblastoma). SHH, *PTC*, *SMO*, and *GLI1* mRNA expression was seen in all samples. *GAPDH* was run as a control to ascertain the integrity of mRNA/cDNA. The sizes of SHH, *PTC*, *SMO*, *GLI1*, and *GAPDH* PCR products were 233, 144, 140, 412, and 206 bp, respectively.

95°C for 15 min and final elongation at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel at 100 V for 40 min and visualized with ethidium bromide.

Immunohistochemistry

Serial cryostat sections each of 5 µm thick were obtained from each frozen block. The sections were fixed in cold acetone for 10 min and washed in cold phosphate-buffered saline (PBS). After treatment with normal rabbit serum for 30 min, the sections were incubated with primary monoclonal antibodies at 4°C overnight. The applied antibodies are listed in Table 1. The standard streptavidin–biotin–peroxidase complex method was performed to bind the primary antibodies with the use of a Histofine SAB-PO Kit (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections for 1–3 min in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide. Nuclei were lightly counterstained with methylgreen. For control studies of the antibodies, the serial sections were treated with PBS and normal goat IgG instead of the primary antibodies and were confirmed to be unstained.

Evaluation of immunostaining and statistical analysis

Immunohistochemical reactivity for SHH, PTC, SMO, and GLI1 was evaluated and classified into three groups: (–)

negative, (+) positive, and (++) strongly positive. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three groups. *P*-values of less than 0.05 were considered to indicate statistical significance.

Results

SHH, PTC, SMO, and GLI1 mRNA expression

RT-PCR analysis identified expression of mRNA transcripts for SHH, PTC, SMO, and GLI1 in all 36 ameloblastomas and 1 malignant ameloblastoma as well as in the nine tooth germ tissues (Fig. 1). The PCR products of SHH, PTC, SMO, and GLI1 were 233, 144, 140, and 412 bp, respectively. There was no distinct difference in SHH, PTC, SMO, and GLI1 mRNA expression among the different types of ameloblastomas or between the malignant and benign ameloblastomas.

Immunohistochemical reactivity for SHH, PTC, SMO, and GLI1

Immunohistochemical reactivity for SHH, PTC, SMO, and GLI1 was detected in the cytoplasm of cellular components

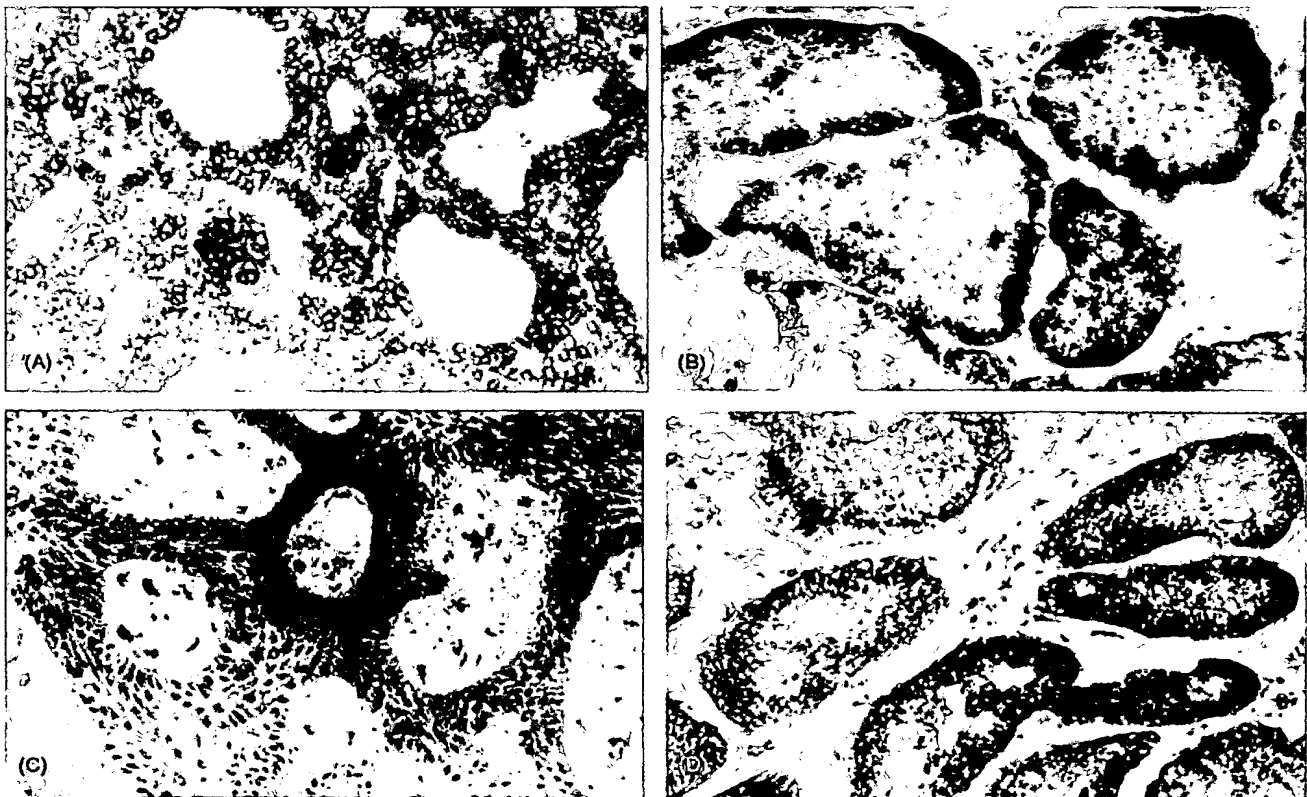


Figure 2 Immunohistochemical reactivity for SHH, PTC, SMO, and GLI1 in ameloblastomas. (A) Plexiform ameloblastoma showing stronger SHH reactivity in peripheral cuboidal cells than in central polyhedral cells. Stromal cells are not reactive ($\times 190$). (B) Follicular ameloblastoma showing stronger PTC reactivity in peripheral columnar cells than in central polyhedral cells. Stromal cells are weakly reactive ($\times 185$). (C) Plexiform ameloblastoma showing SMO reactivity in tumor cells and stromal fibroblasts ($\times 155$). (D) Follicular ameloblastoma showing stronger GLI1 reactivity in peripheral columnar cells than in central polyhedral cells. Stromal cells are weakly reactive ($\times 185$).

Table 2 Immunoreactivity for SHH, PTC, SMO, and GLI1 in tooth germs and ameloblastomas

	SHH			PTC			SMO			GLI1		
	(-)	(+)	(++)	(-)	(+)	(++)	(-)	(+)	(++)	(-)	(+)	(++)
Tooth germ (n = 9)												
Dental lamina	0 (0)	2 (40)	3 (60)	0 (0)	0 (0)	5 (100)	0 (0)	1 (20)	4 (80)	0 (0)	1 (20)	4 (80)
Dental follicle/papilla	0 (0)	9 (100)	0 (0)	9 (100)	0 (0)	0 (0)	0 (0)	2 (22)	7 (78)	0 (0)	9 (100)	0 (0)
Ameloblastoma (n = 36)												
Tumor cells	0 (0)	11 (31)	25 (69)	0 (0)	9 (25)	27 (75)	0 (0)	21 (58)	15 (42)	0 (0)	9 (25)	27 (75)
Stromal cells	8 (22)	28 (78)	0 (0)	0 (0)	30 (83)	6 (17)	0 (0)	10 (28)	26 (72)	0 (0)	27 (75)	9 (25)
Follicular type (n = 24)												
Tumor cells	0 (0)	6 (25)	18 (75)	0 (0)	7 (29)	17 (71)	0 (0)	13 (54)	11 (46)	0 (0)	6 (25)	18 (75)
Stromal cells	4 (17)	20 (83)	0 (0)	0 (0)	20 (83)	4 (17)	0 (0)	8 (33)	16 (67)	0 (0)	17 (71)	7 (29)
Plexiform (n = 12)												
Tumor cells	0 (0)	5 (42)	7 (58)	0 (0)	2 (17)	10 (83)	0 (0)	8 (67)	4 (33)	0 (0)	3 (25)	9 (75)
Stromal cells	4 (33)	8 (67)	0 (0)	0 (0)	10 (83)	2 (17)	0 (0)	2 (17)	10 (83)	0 (0)	10 (83)	2 (17)
Acanthomatous subtype (n = 12)												
Tumor cells	0 (0)	2 (17)	10 (83)	0 (0)	2 (17)	10 (83)	0 (0)	7 (58)	5 (42)	0 (0)	2 (17)	10 (83)
Stromal cells	3 (25)	9 (75)	0 (0)	0 (0)	10 (83)	2 (17)	0 (0)	6 (50)	6 (50)	0 (0)	11 (92)	1 (8)
Granular subtype (n = 2)												
Tumor cells	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)	2 (100)
Stromal cells	0 (0)	2 (100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)	2 (100)	0 (0)	1 (50)	1 (50)
Malignant ameloblastoma (n = 1)												
Tumor cells	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
Stromal cells	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)

Immunohistochemical reactivity: (-), negative; (+), positive; and (++), strongly positive. Values in parentheses denote percentage values.

in both normal and neoplastic odontogenic tissues (Fig. 2; Table 2). In tooth germs, expression of SHH, PTC, and GLI1 was more evident in epithelial cells of the dental laminae than in mesenchymal cells of the dental follicles and dental papillae, and SMO reactivity was marked in both epithelial and mesenchymal cells. (Ameloblastomas showed stronger expression of SHH, PTC, SMO, and GLI1 in peripheral columnar or cuboidal cells than in central polyhedral cells (Fig. 2). In acanthomatous and granular cell ameloblastomas, keratinizing cells and granular cells showed no or little reactivity for these molecules. SMO expression was markedly found in stromal fibroblasts as well as neoplastic cells. Immunoreactivity for SHH, PTC, and GLI1 was weak in stromal cells, and SHH reactivity in stromal cells was not recognized in 8 of 28 ameloblastomas. The malignant (metastasizing) ameloblastoma showed SHH, PTC, SMO, and GLI1 expression patterns similar to those of the benign ameloblastomas. Differences in the immunohistochemical detection of SHH, PTC, SMO, and GLI1 did not reach statistical significance.

Discussion

Sonic hedgehog signals control cell-cell interactions and cell proliferation in tissue patterning during development, and SHH signal transduction is pivotal to embryonic development (14, 15). Targeted gene disruption of *Shh* in mice results in abnormal development of the brain, spinal cord, axial skeleton, and limbs (35). In humans, holoprosencephaly with cyclopia has been attributed to germline mutations with *SHH* (23). Expression of SHH and its signaling molecules have been detected temporally and spatially

during tooth development, indicating a role in early tooth germ initiation and subsequent epithelial-mesenchymal interactions (15-17). In the present study, SHH, PTC, SMO, and GLI1 expression was detected in epithelial and mesenchymal components of human tooth germ tissues in various degrees, suggesting that these molecules are associated with epithelial-mesenchymal interactions during tooth development. Transgenic mice overexpressing *Shh* develop many kinds of tumors, such as basal cell carcinoma, medulloblastoma, and breast carcinoma, suggesting a role of *Shh* in oncogenesis (36). Some human basal cell carcinoma and lung squamous cell carcinoma tissues have shown SHH overexpression (24, 37, 38). In the present study, expression of SHH was detected predominantly in neoplastic cells in benign and metastasizing ameloblastomas. Recently, Heikinheimo et al. (5) have demonstrated by means of a cDNA microarray that the gene transcript for SHH is underexpressed in ameloblastomas. Our results showed that SHH expression in stromal cells of ameloblastomas tended to be low as compared with that in mesenchymal cells of tooth germs.

Germline mutations of *PTC* have been identified to be responsible for basal cell nevus syndrome (BCNS), an autosomal dominant hereditary disease characterized by a variety of clinical problems, including basal cell carcinomas, odontogenic keratocysts, palmar, or plantar pits, ectopic calcification, a spectrum of congenital skeletal anomalies, and various low-frequency neoplasms, such as medulloblastoma and meningioma (21, 22). Homozygous *Ptc* knockout mice die *in utero* during early organogenesis, while heterozygous *Ptc* knockout mice show a number of developmental abnormalities and a high incidence of tumors, similar to human BCNS (39-41). Recent studies have demonstrated somatic

mutations of *PTC* and/or deletions of chromosome 9q22.3, *PTC* locus, in some sporadic tumors, such as basal cell carcinoma, trichoepithelioma, medulloblastoma, meningioma, breast carcinoma, and esophageal carcinoma as well as in patients with BCNS (20, 42–44). *PTC* mutations and deletions of *PTC* locus have been identified in both BCNS-associated and sporadic odontogenic keratocysts (32, 33). Barreto et al. (34) have detected immunoreactivity for PTC protein in ameloblastomas, suggesting that the SHH-signaling pathway might be activated in these odontogenic tumors. In the present study, benign and metastasizing ameloblastomas showed stronger PTC expression in neoplastic cells than in stromal cells, and epithelial PTC expression in ameloblastomas tended to be low as compared with that in tooth germs.

SMO and *GLI1* have been shown to have oncogenic potential (18, 19). Missense mutations of *SMO* have been recognized in sporadic basal cell carcinoma and medulloblastoma, and basal cell carcinoma has shown expression of *SMO* transcript and protein (25, 38). Transgenic mice overexpressing mutant *Smo* have induced basal cell carcinoma (45). Overexpression of *GLI1* has been demonstrated in human basal cell carcinoma, medulloblastoma, glioma, and prostate carcinoma (19, 24), and mice overexpressing *Gli1* have developed basal cell carcinoma as well as other hair follicle-derived tumors, such as trichoepithelioma, cylindroma, and trichoblastoma (46). In the present study, benign and metastasizing ameloblastomas showed *SMO* and *GLI1* expression in neoplastic and stromal cells, and reactivity for *GLI1* was more evident in neoplastic cells than in stromal cells. Epithelial *SMO* expression in these epithelial odontogenic tumors tended to be low as compared with that in dental laminae, and stromal *GLI1* reactivity in ameloblastomas was slightly higher than mesenchymal *GLI1* reactivity in tooth germs. Our examinations detected expression of SHH, PTC, *SMO*, and *GLI1* in benign and metastasizing ameloblastomas at mRNA and protein levels, suggesting that these SHH signaling molecules may play a role in epithelial–mesenchymal interactions and cell proliferation during growth of these epithelial odontogenic tumors. Our results showed no statistically significant difference between normal or neoplastic odontogenic tissues or among the different types of ameloblastomas, and did not clearly indicate that SHH signal transduction had a specific role in oncogenesis of odontogenic epithelium. Further genetic studies of these SHH signaling molecules in odontogenic tumors are needed to establish potential associations between SHH signaling and oncogenesis of odontogenic epithelium.

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Loss of *patched* and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma

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Summary

Medulloblastoma is the most common malignant brain tumor in children. It is thought to result from the transformation of granule cell precursors (GCPs) in the developing cerebellum, but little is known about the early stages of the disease. Here, we identify a pre-neoplastic stage of medulloblastoma in *patched* heterozygous mice, a model of the human disease. We show that pre-neoplastic cells are present in the majority of *patched* mutants, although only 16% of these mice develop tumors. Pre-neoplastic cells, like tumor cells, exhibit activation of the Sonic hedgehog pathway and constitutive proliferation. Importantly, they also lack expression of the wild-type *patched* allele, suggesting that loss of *patched* is an early event in tumorigenesis. Although pre-neoplastic cells

resemble GCPs and tumor cells in many respects, they have a distinct molecular signature. Genes that mark the pre-neoplastic stage include regulators of migration, apoptosis and differentiation, processes crucial for normal development but previously unrecognized for their role in medulloblastoma. The identification and molecular characterization of pre-neoplastic cells provides insight into the early steps in medulloblastoma formation, and may yield important markers for early detection and therapy of this disease.

Key words: Medulloblastoma, Brain tumor, Pre-neoplastic, Patched, Hedgehog, Migration, Differentiation, Mouse

Introduction

Medulloblastoma is a highly malignant pediatric brain tumor (Zakhary et al., 2001). Despite significant improvements in treatment regimens, medulloblastoma remains one of the leading causes of cancer-related death in children under 9 years of age. A deeper understanding of the molecular basis of medulloblastoma is crucial for improving the early diagnosis and treatment of this devastating disease.

Approximately 25% of medulloblastoma cases have mutations in components of the Sonic hedgehog-Patched signaling pathway (Corcoran and Scott, 2001; Ellison et al., 2003). Sonic hedgehog (Shh) is a potent mitogen for cerebellar granule cell precursors (GCPs), the cells from which medulloblastoma is believed to arise (Wechsler-Reya and Scott, 2001; Wechsler-Reya and Scott, 1999). Patched functions as an antagonist of Sonic hedgehog signaling in most tissues (Ingham and McMahon, 2001). People with mutations in the *patched* gene develop Gorlin's syndrome, a disease characterized by basal cell carcinomas, skeletal defects and an increased incidence of medulloblastoma (Hahn et al., 1996; Johnson et al., 1996). Sporadic medulloblastomas also harbor mutations in *patched* and other elements of the Shh pathway (Lam et al., 1999; Raffel et al., 1997; Taylor et al., 2002). Finally, mice heterozygous for mutations in *patched* develop

cerebellar tumors that resemble human medulloblastoma (Goodrich et al., 1997; Hahn et al., 2000).

Although *patched* mutant mice are an important model for medulloblastoma, the molecular and cellular basis of tumorigenesis in these mice remains unclear. Homozygous *patched* knockout mice die during embryonic development with defects in the nervous system, the heart and other tissues (Goodrich et al., 1997). Heterozygotes survive to adulthood, but after 3 months of age, 14–20% develop medulloblastoma (Goodrich et al., 1997; Wetmore et al., 2000). The status of the wild-type *patched* allele in these tumors is controversial: some studies have reported expression of wild-type *patched* in tumor tissue (Romer et al., 2004; Wetmore et al., 2000; Zurawel et al., 2000), whereas others have suggested that the wild-type allele is epigenetically silenced (Berman et al., 2002). Determining whether *patched* is lost – and when during tumorigenesis this loss occurs – is crucial for understanding the mechanisms of medulloblastoma formation.

Studies of *patched* mutant mice suggest that cerebellar abnormalities precede the appearance of tumors. While one-sixth of these animals develop medulloblastoma at 3–6 months of age, more than half have regions of ectopic cells in their cerebella at 4–6 weeks of age (Corcoran and Scott, 2001; Goodrich et al., 1997; Kim et al., 2003). These cells resemble normal GCPs in terms of morphology and location on the

surface of the cerebellum, and have therefore been described as remnants of the external germinal layer (EGL) from which GCPs originate. However, the presence of these cells in mice that are destined to develop medulloblastoma raises the possibility that they may represent a pre-neoplastic stage of tumorigenesis. Determining whether these cells are merely normal cells that persist into adulthood, or whether they are partially transformed cells on their way to becoming tumors has important implications for our understanding of granule cell development and tumorigenesis.

To gain insight into the early stages of medulloblastoma formation, we have isolated ectopic cerebellar cells from *patched* mutant mice and studied their molecular and functional characteristics. Our studies demonstrate that these cells share many properties with tumor cells: they express markers of the granule cell lineage, they exhibit activation of Shh target genes and they proliferate extensively in vitro. In addition, we show that these cells (and tumor cells) completely lack expression of the wild-type *patched* allele, suggesting a mechanism by which these cells maintain active hedgehog signaling. Finally, microarray analysis reveals that these cells have a unique pattern of gene expression that more closely resembles tumor cells than GCPs. Thus, it is likely these cells represent a distinct, pre-neoplastic stage of tumorigenesis. Genes that are differentially expressed at the pre-neoplastic stage include regulators of cell migration, survival and differentiation. Our studies suggest that loss of *patched* expression and dysregulation of these processes may be crucial early events in the development of medulloblastoma.

Materials and methods

Animals

Patched heterozygous mice (Goodrich et al., 1997) were obtained from Matthew Scott's laboratory at Stanford (CA, USA) and maintained by breeding with 129X1/SvJ mice from the Jackson Laboratories (Bar Harbor, ME). Math1-green fluorescent protein (Math1-GFP) transgenic mice (Lumpkin et al., 2003) were provided by Jane Johnson at UT Southwestern Medical Center (Texas, USA). Math1-GFP/*patched*^{+/-} mice were generated by crossing *patched* heterozygotes with Math1-GFP mice, and then backcrossing to Math1-GFP mice three times before further analysis. All mice were maintained in the Cancer Center Isolation Facility at Duke University Medical Center.

Histological staining

To detect expression of β -galactosidase in intact cerebellum, tissue was isolated from adult wild-type or *patched*^{+/-} mice (6- to 12-weeks old) and fixed in 4% paraformaldehyde (PFA) at 4°C. After fixation, tissues were permeabilized in buffer containing 0.01% deoxycholate and 0.02% IGEPAL CA-630 (both from Sigma, St Louis, MO, USA) for 10 minutes. Tissues were washed and stained overnight with X-gal reaction mixture containing 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, 0.4 mg/ml X-galactoside 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, Sigma) in dimethyl sulfoxide, and 1 mM MgCl₂ in phosphate-buffered saline.

To compare expression of β -galactosidase and GFP in sections from Math1-GFP/*patched*^{+/-} mice, cerebella were fixed in 4% PFA, cryoprotected in 25% sucrose, embedded in Tissue Tek-OCT (Sakura Finetek, Torrance, CA, USA) and cryosectioned sagittally at a thickness of 10 μ m. One set of sections (for detection of GFP) was post-fixed for 10 minutes in 2% PFA and immediately mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham,

AL, USA). Adjacent sections were stained with X-gal as described above, counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA, USA) and mounted in Fluoromount G. Fluorescent (GFP) and bright-field (X-gal) images were acquired using a Nikon TE200 inverted fluorescent microscope and Openlab software (Improvision, Lexington, MA, USA).

For histochemical analysis of neonatal cerebellum, pre-neoplastic lesions and tumors, cerebella were fixed overnight in 10% formalin, transferred to 70% ethanol, paraffin wax-embedded and sectioned at 5 μ m. Sections were stained with Hematoxylin and Eosin (Sigma).

Isolation of granule cell precursors, pre-neoplastic cells and tumor cells

Granule cell precursors (GCPs) were isolated from 7-day-old (P7) *patched*^{+/-} mice; pre-neoplastic cells were obtained from 6-week-old *patched* mutants; and tumor cells were obtained from 10- to 25-week-old *patched* mutants displaying physical and behavioral signs of medulloblastoma. Cells were isolated from each source using a protocol described in (Wechsler-Reya and Scott, 1999). Briefly, cerebella were digested in solution containing 10 U/ml papain (Worthington, Lakewood, NJ, USA) and 250 U/ml DNase (Sigma), and triturated to obtain a cell suspension. This suspension was centrifuged through a step gradient of 35% and 65% Percoll (Amersham Biosciences, Piscataway, NJ, USA), and cells were harvested from the 35%-65% interface. Cells were resuspended in serum-free culture medium consisting of Neurobasal containing B27 supplement, sodium pyruvate, L-glutamine and penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, USA), and counted on a hemacytometer. Cells used for RNA isolation were centrifuged and flash frozen in liquid nitrogen. For proliferation assays or immunostaining, cells were plated on poly-D-lysine (PDL)-coated tissue culture vessels and incubated in serum-free culture medium.

Flow cytometry and immunofluorescence

To detect β -galactosidase activity in isolated GCPs, pre-neoplastic cells and tumor cells, cells purified as described above were stained with fluorescein di- β -galactopyranoside (FDG, Marker Gene Technologies, Eugene, OR, USA) for 2 minutes at 37°C. Cells were washed, incubated for 30 minutes on ice and analyzed on a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA). As a control for non-specific FDG staining, GFP⁻ cells were isolated by fluorescence-activated cell sorting (FACS) from Math1-GFP/*patched*^{+/-} mice and stained in the same manner. These cells are not hedgehog responsive, and therefore express low levels of the mutant *patched* allele and low levels of β -galactosidase.

To detect expression of surface markers, cells were stained for 1 hour with primary antibodies, washed, stained for 30 minutes with secondary antibodies, and then analyzed by flow cytometry. To detect expression of intracellular markers, cells were plated (1 million cells/well) on PDL-coated coverslips in 24-well plates, and allowed to adhere for 4-6 hours before fixation with 4% PFA. Cells were stained overnight with primary antibodies, washed, stained with secondary antibodies for 2 hours at room temperature, and then mounted in Fluoromount G. Immunofluorescence was detected using a Nikon TE200 inverted microscope and Openlab software.

Antibodies used for flow cytometry and immunofluorescence included the following: nestin and GFAP (both from BD-Pharmingen, San Diego, CA, USA); O4, A2B5, polysialated (PSA)-NCAM and Zic-1 (all from Chemicon, Temecula, CA, USA); TUJ1 (Covance, Berkeley, CA, USA); and 13A4 anti-prominin/CD133 (a generous gift of Wieland Huttner and Denis Corbeil, Max Planck Institute, Dresden, Germany).

Proliferation assays

Cerebellar cells isolated as described above were resuspended in serum-free medium (Neurobasal + supplements) and transferred to PDL-coated 96-well plates, at a density of 2×10^5 cells/well. Cells

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were pulsed immediately with tritiated thymidine (methyl- ^3H -Td, Amersham, Arlington Heights, IL, USA) and cultured for 18 hours. Following culture, cells were harvested onto filters using a Mach III Manual Harvester 96 (Tomtec, Hamden, CT, USA) and the amount of incorporated radioactivity was quantitated by liquid scintillation spectrophotometry using a Wallac MicroBeta microplate scintillation counter (Perkin Elmer, Boston, MA, USA).

RNA isolation and real-time RT-PCR

To isolate total cytoplasmic RNA from GCPs, pre-neoplastic cells and tumor cells, snap-frozen cell pellets were lysed in buffer containing 0.5% IGEPAL CA-630, digested with Proteinase K, extracted with phenol:chloroform:isoamyl alcohol, and precipitated with ethanol. RNA was purified using RNeasy columns (Qiagen, Valencia, CA, USA) and treated with DNase I (DNA-free, Ambion, Austin, TX, USA) to remove genomic DNA. RNA concentration was determined using the RiboGreen fluorescent dye (Molecular Probes, Eugene, OR, USA) with a TD-700 fluorometer (Turner BioSystems, Sunnyvale, CA, USA).

For real-time RT-PCR analysis, first-strand cDNA was synthesized using equivalent amounts of total RNA (0.1–1 μg) in a 20 μl reverse-transcriptase reaction mixture (Invitrogen). Real-time PCR reactions were performed in triplicate using a 25 μl mixture containing iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), water, primers and 1 μl of cDNA. Gene-specific primers were used for: *Nmyc*, *cyclin D1*, *Gli1*, *Pax6*, *Unc5h3* (*Unc5c*), *Atf3*, *osteopontin* (*Spp1*), *Bag3*, *Foxf2*, *Klf4* and *Neurod1*; sequences for these are available upon request. Real-time quantitation was performed using the BIO-RAD iCycler iQ system (BioRad). Serial tenfold dilutions of cDNA were used as a reference for the standard curve calculation. Raw data were normalized based on expression of *actin*.

For analysis of wild-type and mutant *patched* expression, cells were isolated from Math1-GFP/*patched*^{+/−} mice as described above, and then FACS-sorted to obtain pure populations of GFP⁺ cells. RNA was isolated using the RNeasy-Micro kit (Ambion), DNase-treated, quantitated, converted to cDNA and subjected to real-time PCR analysis as described above. Primers used to amplify *patched* were as follows: exons 2–3, 5'-GGC AAG TTT TTG GTT GTG GGT C-3' (forward) and 5'-CCT CTT CTC CTA TCT TCT GAC GGG-3' (reverse); and exons 7–9, 5'-CAT TGG CAG GAG GAG TTG ATT G-3' (forward) and 5'-GCA CCT TTT GAG TGG AGT TTG G-3' (reverse).

Microarray hybridization and analysis

RNA from GCPs, pre-neoplastic cells and tumor cells (isolated as described above, but not FACS-sorted), and from normal adult cerebellum (not dissociated), was converted to cDNA using the Superscript Choice cDNA kit (Invitrogen) and a T7-dT(24) primer (Genset/Prologo, Boulder, CO, USA). cRNA was generated using a T7-transcription/labeling kit from Enzo Life Sciences and hybridized to Affymetrix U74Av2 chips (Affymetrix, Santa Clara, CA, USA). Chips were scanned, and hybridization data were acquired using Affymetrix Suite 5.0 software. Affymetrix CEL files were normalized and quantified using Bioconductor software with the gcRMA model to quantify gene expression levels (Gentleman and Carey, 2002). Unsupervised principal components analysis (PCA) was used to identify the relationships among normal adult cerebellum, GCPs, pre-neoplastic cells and tumor cells based on expression profiles.

To identify genes that were differentially expressed among GCPs, pre-neoplastic cells and tumor cells, supervised analysis was carried out. A gene-by-gene analysis of variance (ANOVA) model with three groups (GCP, pre-neoplastic, tumor) was used to fit the log₂-transformed intensities. To correct for multiple comparisons, the nominal *P*-value was adjusted using the false discovery rate (Benjamini and Hochberg, 1995). Genes were considered to be differentially expressed if they satisfied all of the following criteria: a difference in expression greater than 1.9-fold between any two groups; a maximum

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absolute intensity difference larger than 32 units; and an adjusted *P*-value <0.01. There were 118 genes that met these criteria. The identities of differentially expressed genes were verified by integrating data from the Affymetrix and Unigene databases. Gene functions were determined using information from Gene Ontology, Unigene, LocusLink and PubMed databases. Clustering was performed with Cluster and Treeview (Eisen et al., 1998). All statistical analysis was performed using R-1.7 software (Dalgaard, 2002). Results were visualized with Spotfire 6.0 (Somerville, MA, USA).

Immunohistological validation of microarray genes

Tissues were processed as described for the X-gal histological staining in the methods above for P7, 6-week-old, and 10- to 25-week-old *patched* mutant mice with tumors. PFA-fixed frozen sections were rehydrated in Tris-buffered saline and permeabilized with 2% Triton X-100 (Sigma) for 10 minutes. Sections were stained overnight at 4°C with rabbit polyclonal antibodies specific for *Zic3* (Chemicon), *Necdin* (Upstate, Waltham, MA, USA) or *Hsp105* (Biovision, Mountain View, CA, USA), or with mouse monoclonal antibodies specific for *Pax6* (R&D Systems, Minneapolis, MN, USA). Antibody staining was detected using the EnVision+ Peroxidase-DAB system (Dako Cytomation, Carpinteria, CA, USA), as described in the manufacturer's protocol. Sections were counterstained with Harris Hematoxylin (Sigma) and mounted using Vectamount (Vector Laboratories).

Results

Detection of ectopic cerebellar cells in adult *patched* mutant mice

Patched mutant mice develop cerebellar tumors that resemble human medulloblastoma. These tumors are located on the surface of the cerebellum and express high levels of β -galactosidase (Fig. 1C). [When the *patched* mutant allele was generated, a portion of the *patched* gene was replaced with the β -galactosidase coding sequence (Goodrich et al., 1997); thus, staining with the β -galactosidase substrate X-gal reflects increased expression of the mutant *patched* allele.] Tumors occur in 15–20% of mice between 10 and 25 weeks of age. However, at 3–6 weeks of age, before any of the mice exhibit physical manifestations of tumor development, more than half have foci of ectopic β -galactosidase-expressing cells on the surface of their cerebellum (arrowheads in Fig. 1B). At early ages, several foci are commonly found in each animal; by 6–8 weeks, most animals have only one or two.

To examine the morphological characteristics of these cells, we sectioned cerebella from *patched* mutant animals and stained them with Hematoxylin and Eosin. At postnatal day 7 (P7), the cerebellum of *patched* mutant mice contains granule cell precursors (GCPs) on its surface and is indistinguishable from the cerebellum of wild-type mice (compare Fig. 2A with 2D). But by three weeks of age, wild-type and mutant cerebella are clearly different. In wild-type animals, all GCPs have differentiated and migrated inward, and the surface of the cerebellum consists mostly of neuronal processes (Fig. 2B,C). By contrast, 3-week-old *patched* mutant mice often have multiple regions of cells that have failed to migrate and instead remain on the surface of the cerebellum (Fig. 2E). These cells are densely packed and have a high nucleus:cytoplasm ratio (properties shared by both GCPs and tumor cells). In addition, staining with antibodies to the proliferation marker Ki67 reveals that a large percentage of these cells are in cycle (data not shown). Foci of ectopic cells are also seen in 6-week-old

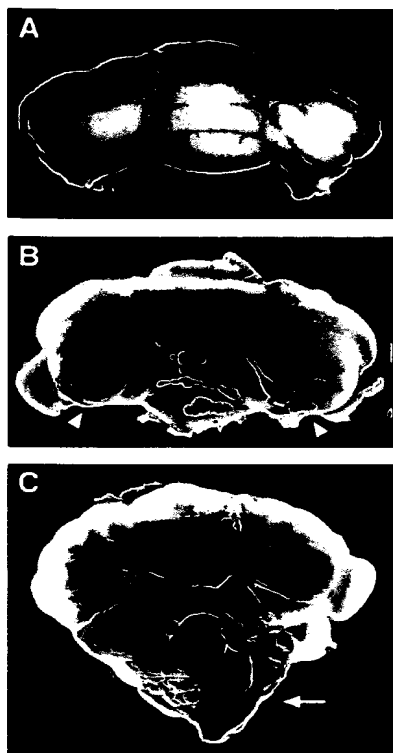


Fig. 1. The majority of *patched* mutant mice have ectopic cells in their cerebellum before they develop tumors. Cerebella from a 6-week-old wild-type mouse (A), a 6-week-old *patched*^{+/-} mouse (B) and a 12-week-old *patched*^{+/-} mouse with a tumor (C) were fixed and stained with X-gal. Tumors (arrow in C) are found in 15–20% of mutant mice between 10–25 weeks of age; these tumors express high levels of the mutant *patched* allele, which carries the β -galactosidase gene. At earlier ages, >50% of *patched* mutant mice have ectopic β -galactosidase-expressing cells (arrowheads in B). Background X-gal staining was not detected in the cerebellum of adult wild-type mice (A).

patched mutant animals (Fig. 2F), but are rarely observed beyond 10 weeks of age; at that age, *patched* mutant mice either show no cerebellar abnormalities or have large tumors that encompass the outer surface of their cerebellum (Fig. 2G). Thus, the majority of *patched* mutant mice have proliferating cells on the surface of their cerebellum well before they develop medulloblastoma.

The fact that the ectopic cells in *patched* mutants resemble tumor cells in terms of morphology, location and β -galactosidase expression – and occur in animals that are destined to develop tumors – suggests that they might represent an early, pre-neoplastic stage of medulloblastoma. Further studies of these cells (described below) strongly support this hypothesis; we therefore refer to them as ‘pre-neoplastic cells’ in the remainder of the text.

Pre-neoplastic cells express markers of immature granule cells

Previous studies have suggested that medulloblastoma arises

from granule cell precursors (Buhren et al., 2000; Kadin et al., 1970; Miyata et al., 1998). To determine whether pre-neoplastic cells express markers of the granule cell lineage, we crossed *patched*^{+/-} mice with Math1-GFP mice (Lumpkin et al., 2003). In the developing cerebellum, Math1 is a specific marker for proliferating granule cell precursors (Ben-Arie et al., 1996; Helms and Johnson, 1998). As GCPs cover the surface of the cerebellum during the first 2–3 weeks of life, the cerebellum of Math1-GFP mice is intensely fluorescent during this period (Fig. 3A). By 6 weeks of age, all GCPs have migrated inward and differentiated into mature granule neurons; thus, GFP is not expressed in the cerebellum of adult Math1-GFP mice (Fig. 3B). However, in Math1-GFP/*patched*^{+/-} mice, regions of ectopic GFP⁺ cells are frequently seen in the cerebellum at 6 weeks of age (Fig. 3C,E). Tumors arising in these mice are also strongly GFP⁺ (Fig. 3D). These data suggest that pre-neoplastic cells and tumor cells express Math1 and are derived from the granule cell lineage.

To confirm that the GFP-expressing cells in adult Math1-GFP/*patched*^{+/-} mice are the same as the ectopic β -galactosidase-expressing cells described above (Fig. 1), we sectioned cerebella from these mice and stained them with X-gal. As shown in Fig. 4A–C, GFP⁺ regions of cerebellum from 6-week-old mice show strong X-gal staining. Likewise, tumors from Math1-GFP/*patched*^{+/-} mice show similar patterns of GFP expression and X-gal staining (Fig. 4D–F). Thus, in Math1-GFP/*patched*^{+/-} mice, pre-neoplastic cells and tumor cells can be clearly identified by their expression of β -galactosidase and GFP. To gain insight into the early stages of tumorigenesis in *patched* mutant mice, we sought to isolate pre-neoplastic and tumor cells, and study their molecular and functional characteristics.

Isolation of pre-neoplastic cells and tumor cells from adult *patched* mutant mice

To isolate pre-neoplastic cells and tumor cells from *patched* mutant mice, we used a method originally developed for purifying neonatal GCPs (Oliver et al., 2003; Wechsler-Reya and Scott, 1999). This method selects for small, dense cells without processes, and destroys the majority of process-bearing neurons and glia that make up the normal adult cerebellum. Thus, when we used it to isolate cells from 6-week-old wild-type mice, we obtained an average of only 0.5 million cells per animal ($n=10$, range 0.2–0.9 million), and none of these cells had a morphology similar to that of GCPs. By contrast, when we performed this procedure on 6-week-old *patched* mutant mice, we obtained an average of 4.3 million GCP-like cells ($n=43$, range: 0.5–50 million) (Fig. 5A). Among *patched* mutant mice, 84% had more than 0.9 million cells, the highest number obtained from a wild-type mouse at this age. At 10–25 weeks of age, 16% of *patched* mutant mice had readily identifiable tumors. From these animals, we could isolate 50–600 million GCP-like cells (Fig. 5B). The remaining animals had no discernable abnormalities in their cerebellum, and we never obtained more than 2 million cells from them. These studies demonstrate that pre-neoplastic cells and tumor cells can be isolated from the cerebellum of *patched* mutant mice.

To verify that the cells we isolated from *patched* mutant mice represent the β -galactosidase-expressing cells identified by whole-mount staining of the cerebellum (Fig. 1), we stained

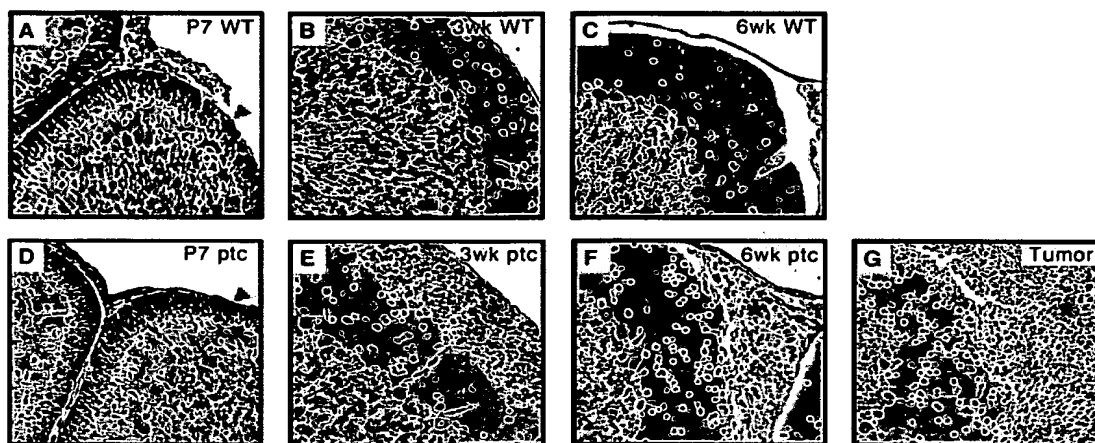


Fig. 2. Ectopic 'pre-neoplastic' cells resemble tumor cells. Cerebella were isolated from wild-type mice (A-C) and *patched*^{+/−} mice (D-G) at 7 days (A,D), 3 weeks (B,E) 6 weeks (C,F) or 12 weeks (G) of age. Tissues were paraffin wax-embedded, sectioned and stained with Hematoxylin and Eosin. At 7 days of age, wild-type and *patched* mutant cerebella are indistinguishable, with densely-packed GCPs on the surface (arrowheads in A and D). Note the presence of ectopic cells on the outside of the cerebellum in adult *patched* mutant mice (asterisks in E and F) but not in wild-type mice (B,C). These cells are present in the majority of *patched* mutants, and resemble tumor cells (asterisk in G) in terms of size, morphology and location.

these cells with a fluorescent β -galactosidase substrate (FDG) and analyzed them by flow cytometry. As shown in Fig. 5C, the pre-neoplastic cells isolated from 6-week-old *patched* heterozygotes and tumor cells isolated from 16-week-old animals were 89–96% FDG⁺. Moreover, when cells were isolated from Math1-GFP/*patched*^{+/−} mice, 87% of pre-neoplastic cells and 95% of tumor cells were found to be GFP⁺. Thus, freshly isolated pre-neoplastic cells and tumor cells, like their counterparts in situ, express high levels of β -galactosidase and Math1-GFP.

To further examine the phenotype of these cells, we isolated

them and stained them with antibodies specific for various cell types (see Table S1 in supplementary material). Consistent with the expression of Math1-GFP, a large percentage of cells in each population expressed neuronal markers (tubulin β III/TuJ1, polysialated NCAM and Zic1) (Miyata et al., 1998; Yokota et al., 1996). A small number of cells in each population expressed the oligodendrocyte marker O4 (Sommer and Schachner, 1981), suggesting that some cells of this lineage co-purify with GCPs in our isolation procedure. By contrast, very few cells expressed markers of astrocytes (glial fibrillary acidic protein) (Bignami et al., 1972) or neural stem cells (nestin, CD133/prominin) (Sawamoto et al., 2001; Weigmann et al., 1997). These findings support the notion that pre-neoplastic cells are derived from the granule cell lineage.

Pre-neoplastic cells express elevated levels of hedgehog target genes and proliferate in vitro

In *patched* heterozygotes, β -galactosidase activity is a reporter for expression of the mutant *patched* allele. Because *patched* is a target of the hedgehog pathway (Goodrich et al., 1996), the β -galactosidase activity observed in pre-neoplastic cells (Fig. 1B, Fig. 4C) suggests that these cells have increased activation of the hedgehog pathway. To determine whether these cells express elevated levels of other hedgehog target genes, we performed quantitative (real-time) RT-PCR analysis using primers specific for *Gli1*, *cyclin D1* and *Nmyc*, genes

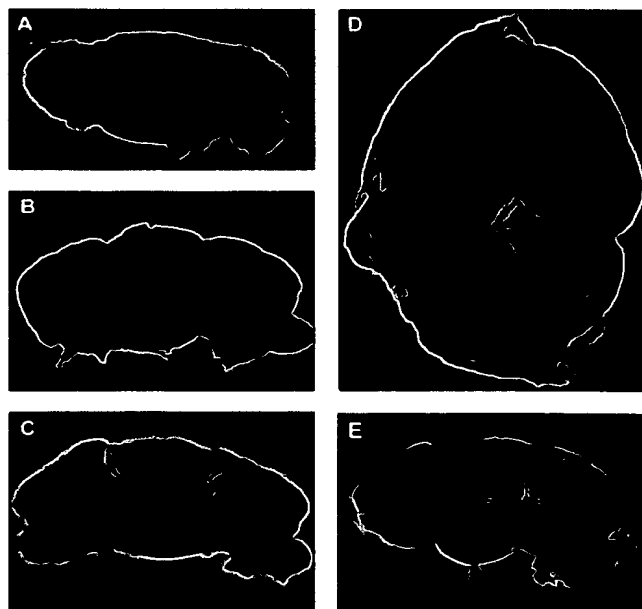


Fig. 3. Pre-neoplastic cells express the granule cell lineage marker Math1. Cerebella from 7-day-old (A) and 6-week-old (B) Math1-GFP transgenic mice, and from 6-week-old (C,E) and 18-week-old (D) Math1-GFP/*patched*^{+/−} mice, were photographed using a Leica MZFLIII microscope equipped with SPOT camera and software. Fluorescent and bright-field images were overlaid using Photoshop. (D) Entire brain, with cerebellum (including tumor) at bottom. Note the GFP-expressing (green) pre-neoplastic and tumor regions (C-E) in *patched* mutant mice.

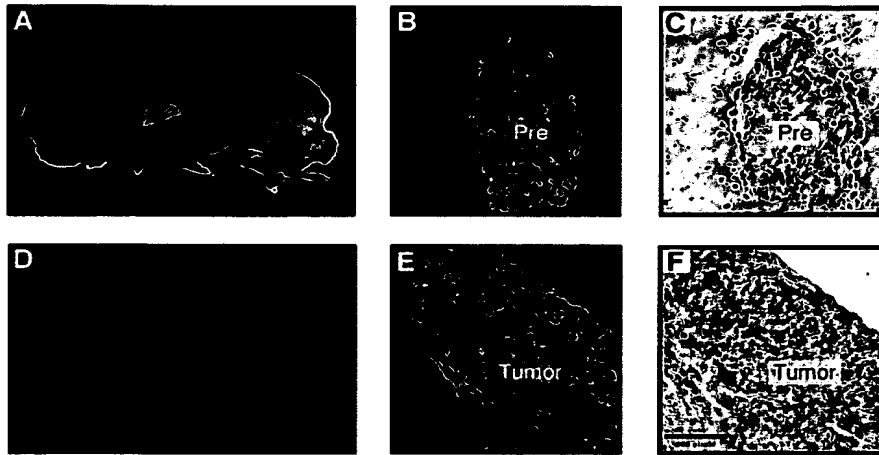


Fig. 4. Co-expression of Math1 and β -galactosidase in pre-neoplastic cells and tumor cells. Cerebella were isolated from 6-week-old (A-C) and 12-week-old (D-F) tumor bearing Math1-GFP/*patched*^{+/-} mice. Intact cerebella (A,D) were photographed using a Leica MZFLIII microscope and then fixed, frozen and cryosectioned. One set of sections was mounted and photographed using a fluorescent microscope to detect GFP (B,E); adjacent sections were stained with X-gal, mounted and photographed under bright field (C,F). Note the correlation between GFP (green, indicative of Math1 expression) and X-gal staining (blue, indicative of mutant *patched* expression) in pre-neoplastic (B,C) and tumor-containing (E,F) regions.

previously identified as hedgehog targets in GCPs (Kenney et al., 2003; Kenney and Rowitch, 2000; Oliver et al., 2003; Wechsler-Reya and Scott, 1999). As a reference for the level of mRNA associated with hedgehog pathway activation, we compared the levels of these genes to the levels in resting granule cell precursors (cultured for 24 hours in the absence of Shh). As shown in Fig. 6, freshly isolated GCPs, pre-neoplastic cells and tumor cells all exhibit significantly elevated levels of Shh target genes when compared with resting cells (3- to 5-fold for *Nmyc*, 4- to 9-fold for *cyclin D1*, 2000- to 6000-fold for *gli1*). For each gene, the levels in GCPs, pre-neoplastic cells and tumor cells were comparable to or higher than the levels in cells that had been stimulated with Shh for 24 hours (data not shown). These data suggest that the Shh signaling pathway is activated in pre-neoplastic cells.

In granule cell precursors, Shh pathway activation is associated with proliferation (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). To determine whether pre-neoplastic cells are proliferating, we measured thymidine incorporation in these cells following isolation. As shown in Fig. 6D, pre-neoplastic cells incorporate thymidine at levels

comparable to freshly isolated GCPs and tumor cells. Thus, the pre-neoplastic stage is characterized by persistent hedgehog pathway activation and proliferation.

Pre-neoplastic cells lack expression of wild-type *patched*

Our studies indicated that GCPs, pre-neoplastic cells and tumor cells all exhibit hedgehog pathway activation and proliferation when they are isolated. In GCPs, expression of Shh targets reflects exposure to Shh in vivo prior to isolation (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). In tumor cells, Shh pathway activation has been suggested to result from silencing of the wild-type *patched* allele (Berman et al., 2002), although some groups have reported persistent expression of wild-type *patched* in tumors (Romer et al., 2004; Wetmore et al., 2000; Zurawel et al., 2000). The status of wild-type *patched* in pre-neoplastic cells has not been investigated. Our ability to isolate pre-neoplastic cells and tumor cells to near-homogeneity allowed us to examine *patched* expression in these populations in the absence of contaminating (non-tumor) cells.

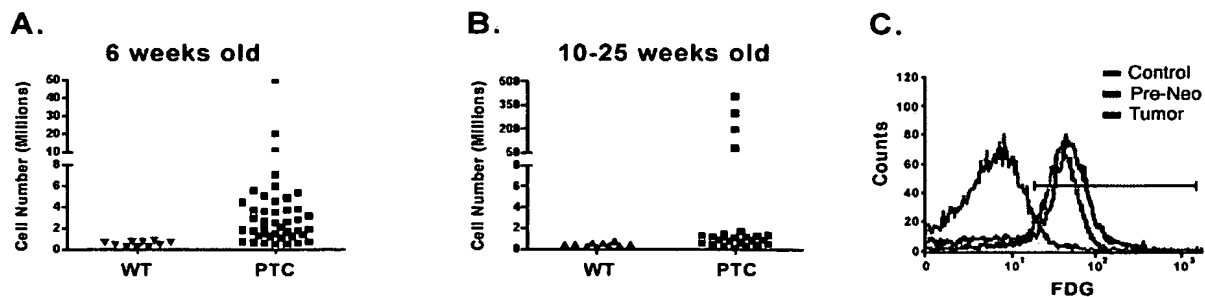


Fig. 5. Pre-neoplastic cells can be isolated from the cerebellum of *patched* mutant mice. Cells were isolated from the cerebellum of wild-type and *patched*^{+/-} mice by enzymatic dissociation followed by Percoll gradient centrifugation, and viable cells were counted after Trypan Blue staining. (A) The average yield for 6-week-old wild-type mice was 0.53 ± 0.25 million cells. For *patched* heterozygotes of the same age, the average yield was 4.3 million cells, with 84% of animals having more than 0.9 million cells (the maximum number seen in wild-type mice). (B) Among older *patched* mutants (10-25 weeks), 16% had tumors containing 50-600 million cells; the remainder had fewer than 2 million cells. (C) Non-granule cell precursors (GFP⁻ cells from neonatal Math1-GFP/*patched*^{+/-} mice), pre-neoplastic cells and tumor cells were stained with the fluorescent β -galactosidase substrate FDG and analyzed by flow cytometry. Relative fluorescence of non-GCPs (blue), pre-neoplastic cells (pink) and tumor cells (purple) is shown. The horizontal black line indicates the range of fluorescence considered to be positive (i.e. above background); 89% of pre-neoplastic cells and 96% of tumor cells exhibited fluorescence within this range.

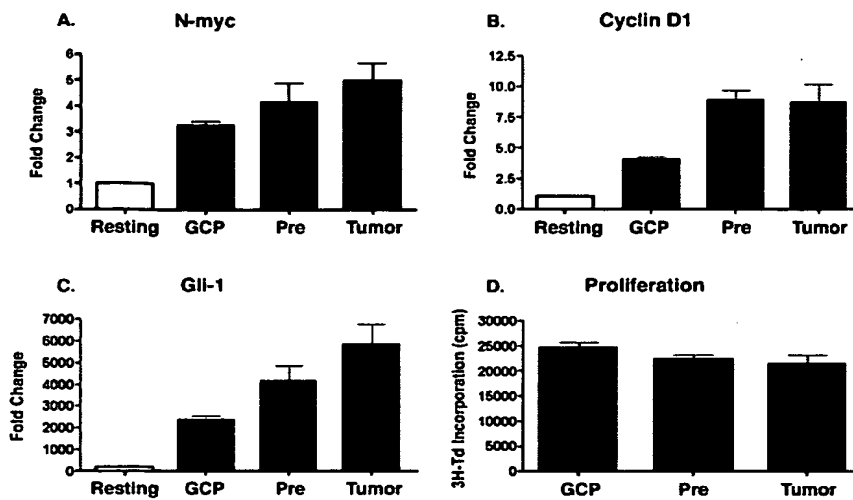


Fig. 6. Pre-neoplastic cells exhibit hedgehog pathway activation and proliferation. (A-C) Expression of hedgehog target genes. RNA was purified from freshly isolated GCPs, pre-neoplastic cells and tumor cells, and from GCPs cultured for 24 hours in the absence (resting) or presence (stimulated) of Sonic hedgehog (3 μ g/ml Shh-N). Equivalent amounts of RNA were reverse transcribed and subjected to real-time PCR analysis using primers for *Nmyc* (A), *cyclin D1* (B) or *gli1* (C). Expression levels were normalized to *actin* and divided by the levels in resting GCPs to calculate fold induction. Data represent means of three samples of each cell type $\pm$ s.e.m. (D) Proliferation of GCPs, pre-neoplastic cells and tumor cells. Cells were pulsed with tritiated thymidine immediately after isolation, and then cultured for 18 hours in serum-free media before being harvested and assayed for thymidine incorporation. Data represent means of triplicate samples $\pm$ s.e.m., and are representative of 16 experiments.

We analyzed *patched* expression in GCPs, pre-neoplastic cells and tumor cells by real-time RT-PCR. To distinguish between wild-type and mutant *patched* transcripts, we used two sets of primers: one derived from exons 7-9, which are present in both the wild-type and mutant alleles, and another derived from exons 2-3, which can only amplify sequences present in the wild-type allele (see Fig. 7A). As shown in Fig. 7B, transcripts containing exons 7-9 were comparably expressed in GCPs, pre-neoplastic cells and tumor cells. By contrast, transcripts containing exons 2-3 (wild-type *patched*) were found in GCPs but were absent from the majority of pre-neoplastic cells and tumor cells (Fig. 7C). Similar results were seen using a pair of primers within exon 2. Overall, loss of wild-type *patched* was observed in 6 out of 7 pre-neoplastic samples and 13 out of 13 tumor samples, but was never seen in GCPs or in normal adult cerebellum from *patched*^{+/-} mice (see Fig. S1 in supplementary material).

These results suggest that the Shh pathway activation and proliferation seen in pre-neoplastic and tumor cells results from de-repression of the pathway due to loss of *patched*. Moreover, they indicate that loss of *patched* occurs at an early stage of tumorigenesis, well before cells have committed to becoming full-blown tumors.

Pre-neoplastic cells have a unique molecular signature

Together, the above data demonstrate that GCPs, pre-neoplastic cells and tumor cells are remarkably similar in terms of morphology, cell lineage, (hedgehog pathway activation, and proliferation.) However, all *patched* mutant mice have GCPs, whereas only a subset have detectable pre-neoplastic cells and only 16% develop tumors. Thus, despite their similarities, GCPs, pre-neoplastic cells and tumor cells must differ at a molecular level. To identify molecular differences between

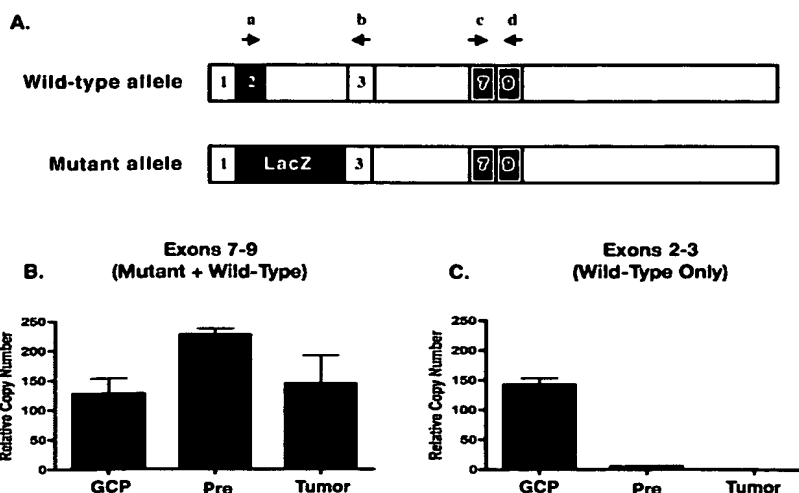


Fig. 7. Pre-neoplastic cells do not express wild-type *patched*. (A) Primers used to distinguish wild-type and mutant *patched* transcripts. The predicted structures of wild-type and mutant *patched* transcripts are shown. In the mutant allele, a portion of exon 1 and all of exon 2 have been replaced with the β -galactosidase coding sequence (*lacZ*). Thus, sequences within this region (detected by primers a and b) should only be present in wild-type transcripts. Sequences within exons 7 and 9 (detected by primers c and d) should be present in both wild-type and mutant transcripts. (B,C) Expression of wild-type and mutant *patched*. RNA from FACS-sorted GCPs, pre-neoplastic cells and tumor cells was subjected to real-time PCR analysis using primers c and d (exons 7-9, B) or primers a and b (exons 2-3, C). Expression levels were normalized to *actin*. Data represent means of three samples $\pm$ s.e.m. Loss of wild-type *patched* expression was observed in six out of seven pre-neoplastic samples and 13 out of 13 tumor samples; see Fig. S1 in supplementary material for details.

GCPs, pre-neoplastic cells and tumor cells, we compared gene expression in these populations using DNA microarrays. RNA from GCPs, pre-neoplastic cells and tumor cells was labeled and hybridized to Affymetrix U74Av2 GeneChips, and data were analyzed using Bioconductor software (Gentleman and Carey, 2002) (see Materials and methods for details).

To determine how closely related these samples were in terms of gene expression, we performed unsupervised principal components analysis (PCA). When the analysis included normal adult cerebellum (Fig. 8A), GCPs, pre-neoplastic cells and tumor cells appeared highly similar to one another and quite distinct from adult cerebellum. This is not surprising, as adult cerebellum consists of postmitotic neurons and glia of various lineages whereas GCPs, pre-neoplastic cells and tumor cells are all proliferating cells derived from the granule cell lineage. By contrast, when GCPs, pre-neoplastic cells and tumor cells were compared directly to one another, each population exhibited a unique pattern of gene expression (Fig. 8B). Hierarchical clustering of the samples (Fig. 8C) supported this conclusion, and indicated that pre-neoplastic cells and tumor cells resemble one another more closely than either population resembles GCPs. Thus, GCPs, pre-neoplastic cells and tumor cells are readily distinguishable at a molecular level.

To characterize the differences in gene expression between GCPs, pre-neoplastic cells and tumor cells, we performed analysis of variance (ANOVA). This analysis identified 118 genes whose expression differed more than 1.9-fold (with a statistical confidence of $P < 0.01$) between populations (see Fig. 9). Expression of 75 genes differed between GCPs and pre-neoplastic cells, and 80 genes distinguished GCPs from tumors. Only 34 genes changed between pre-neoplastic and tumor cells, consistent with the notion that these populations are more closely related to one another than to GCPs.

A large proportion of the differentially expressed genes were associated with three biological processes: cell migration, (cell stress/apoptosis) and (differentiation) (Table 1). Among the regulators of migration were extracellular matrix molecules (Osteopontin, Pleiotrophin, Procollagen IV), cell surface receptors (Unc5h3, Protein tyrosine phosphatase receptor type z) and transcription factors (Pax6). Genes associated with

protection from cell stress included the transcription factor Atf3, the chaperone regulator Bag3, and several heat shock proteins. Finally, genes involved in cell fate and differentiation included transcription factors such as Foxf2, Klf4, Sox2, Neurod1 and Zic3. Significant differences in expression were also seen in genes encoding signaling molecules (G-protein coupled receptor 37-like 1, Gab1, Rhon, Calmodulin-like 4), metabolic genes (Carbonic anhydrase 2, Prostaglandin D2 synthase), channels (Aquaporin 4, Kcnd2), and secreted factors (Chemokine ligand 27, Vascular endothelial growth factor C). Finally, a number of sequences resembling endogenous retrovirus-like elements known as intracisternal A particles (IAPs) were found to be overexpressed at the tumor stage.

To confirm the results of our microarray analysis, we isolated RNA from independent samples of GCPs, pre-neoplastic cells and tumor cells and examined expression of representative genes using real-time RT-PCR. As shown in Fig. S2 (see supplementary material), each of these genes (Pax6, Unc5h3, Atf3, Osteopontin, Bag3, Foxf2, Klf4 and Neurod1) showed a distinct expression pattern in GCPs, pre-neoplastic cells and tumor cells, and the changes in expression were consistent with our microarray data. To test whether differentially expressed genes were targets of the Shh pathway, we cultured GCPs in the presence or absence of Shh protein for 12–24 hours and analyzed expression of select genes by real-time RT-PCR (data not shown). None of genes we tested were significantly altered by Shh stimulation, supporting the notion that the differences between GCPs, pre-neoplastic cells and tumor cells are not the result of differential Shh pathway activation. Finally, to determine whether the genes we identified in purified cells were also differentially expressed *in situ*, we used antibodies against the protein products of some of these genes to stain sections of neonatal (P7) cerebellum, pre-neoplastic lesions and tumors. As shown in Fig. 10, expression of these proteins (Zic3, Pax6, Necdin and Hsp105) was significantly different in GCPs, pre-neoplastic cells and tumor cells *in situ*, and mirrored the expression of the corresponding transcripts in purified cells.

Together these studies demonstrate that GCPs, pre-neoplastic cells and tumor cells can be distinguished at a molecular level and are likely to represent distinct stages of

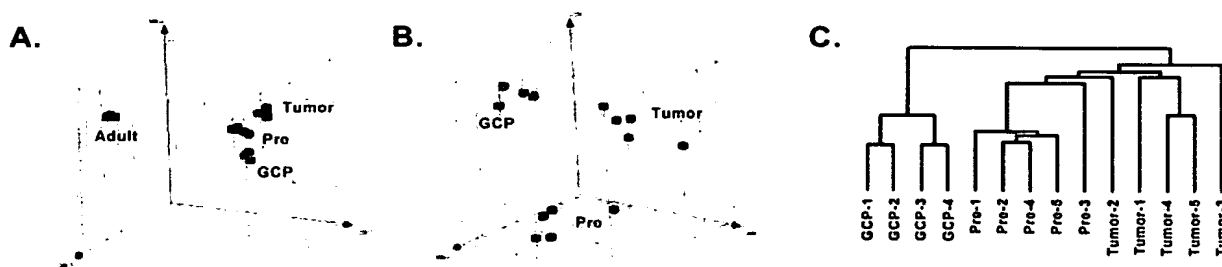


Fig. 8. GCPs, pre-neoplastic cells and tumor cells have distinct gene expression profiles. Gene expression in GCPs (four separate litters), pre-neoplastic cells (five mice), and tumor cells (five mice) from *patched* mutant mice, and adult cerebellum from wild-type mice (four mice), was analyzed using Affymetrix U74Av2 microarrays. Unsupervised principal components analysis (PCA) was used to assess the similarity in gene expression between these samples. (A) PCA plot of all 18 samples indicates that GCPs, pre-neoplastic cells and tumor cells are very similar to one another when compared with normal adult cerebellum. (B) Analysis excluding normal adult cerebellum suggests that compared with one another, GCPs, pre-neoplastic cells and tumor cells each have unique profiles of gene expression. (C) Single-linkage hierarchical clustering of the samples suggests that GCPs, pre-neoplastic cells and tumor cells are distinct, with pre-neoplastic and tumor cells resembling one another more closely than either resembles GCPs.



Fig. 9. Genes that distinguish GCPs, pre-neoplastic cells and tumor cells. Microarray data were subjected to analysis of variance (ANOVA) and genes that changed more than 1.9-fold between GCPs, pre-neoplastic cells and tumor cells (with adjusted *P*-values <0.01 and maximum absolute intensity difference >32 units) were considered differentially expressed. Expression profiles of the 118 differentially expressed genes are shown. Colors represent relative expression level, with red denoting high and green denoting low expression (see gradient at bottom of figure). Genes were clustered based on expression pattern among the three groups.

Table 1. Functions of differentially expressed genes

	Gene symbol	Unigene#	Type of molecule	Pre:GCP	Tumor:GCP
Adhesion/migration					
Secreted phosphoprotein 1 (osteopontin)	spp1, opn	Mm.257330	Extracellular matrix	-18.7	-2.5
Nidogen 2	nid2	Mm.20348	Extracellular matrix	-17.6	-22.1
Unc5 homolog C	unc5h3, unc5c	Mm.24430	Cell surface	-8.4	-9.3
Pleiotrophin	ptn	Mm.3063	Extracellular matrix	-4.4	-3.8
Collagen IV, alpha1	col4a1	Mm.738	Extracellular matrix	-3.8	-2.6
Paired box gene 6	pax6	Mm.3608	Transcription factor	-3.4	-4.7
CD47 (integrin-associated protein)	cd47, iap	Mm.167842	Cell surface	-2.5	-3.2
Protein tyrosine phosphatase, receptor type	ptprz1	Mm.41639	Cell surface	-1.6	-25.8
Gap junction protein a1 (Connexin 43)	gja1, cx43	Mm.4504	Cell surface	-1.4	-6
Platelet activating factor acetylhydrolase	pafah, pla2g7	Mm.9277	Secreted enzyme	-1.2	-5.7
Apoptosis/stress					
Bcl2-associated athanogene 3	bag3	Mm.28373	Chaperone regulator	9	4.1
Activating transcription factor 3	atf3	Mm.2706	Transcription factor	3.9	3
Heat shock protein 105	hsp105	Mm.270681	Chaperone	3.4	2.4
DnaJ (Hsp40) homolog B1	dnajb1	Mm.282092	Chaperone	2	2
DnaJ (Hsp40) homolog B10	dnajb10	Mm.248776	Chaperone	2	-2.5
Metaxin 2	mtx2	Mm.292613	Mitochondrial protein	-3.8	-29.4
Cell fate/differentiation					
FBJ osteosarcoma oncogene B	fosb	Mm.248335	Transcription factor	11.7	43
Forkhead box F2	foxF2	Mm.169989	Transcription factor	6.6	15.5
Kruppel-like factor 4	klf4, gklf	Mm.4325	Transcription factor	4.1	11.6
Cysteine and glycine-rich protein 1	csrp1	Mm.196484	Nuclear protein	3.8	2.9
Zinc finger protein 288	zfp288	Mm.211212	Transcription factor	3.5	2.7
Early growth response 2	egr2, krox20	Mm.290421	Transcription factor	2.7	3
Sry box containing gene 2	sox2	Mm.51994	Transcription factor	-1.7	-7.2
Neurogenic differentiation 1	neuroD1, beta2	Mm.4636	Transcription factor	-2.6	-2.4
Nedrin	ndn	Mm.250919	Nuclear protein	-5	-8.6
Zinc finger protein of the cerebellum 3	zic3	Mm.4265	Transcription factor	-6.7	-11.1

Functions were assigned to genes based on information from Gene Ontology, Unigene, LocusLink and PubMed databases. Representative genes from the three major functional groups are presented. Numbers refer to the fold change in gene expression between GCPs and pre-neoplastic cells (Pre:GCP), and between GCPs and tumor cells (Tumor:GCP).

tumorigenesis. It is important to note that few of the genes that were differentially expressed between GCPs, pre-neoplastic cells and tumor cells were granule cell lineage markers, elements of the cell cycle machinery or known targets of the hedgehog pathway. This is consistent with our previous observation that these cells resemble one another in terms of lineage, (hedgehog pathway activation and proliferation.) By comparing similar populations of cells at different stages of tumorigenesis, we were able to identify other processes and pathways that contribute to medulloblastoma formation. (The fact that pre-neoplastic cells and tumor cells have significant changes in genes that control migration, apoptosis and differentiation suggests that dysregulation of these processes may be crucial for the development of medulloblastoma.)

Discussion

Medulloblastoma is a highly malignant and frequently fatal tumor, but little is known about the early stages of the disease. We have used a mouse model of medulloblastoma to identify a pre-neoplastic stage and define its molecular characteristics. Our data demonstrate that pre-neoplastic cells show elevated hedgehog pathway activation and proliferation, and lack expression of wild-type *patched*. In addition, pre-neoplastic cells have a unique molecular signature characterized by altered expression of genes that regulate migration, apoptosis and differentiation. These studies define a crucial early stage of tumorigenesis and provide insight into its molecular basis.

Pre-neoplastic cells in *patched* mutant mice were first described by Goodrich et al. as 'regions of increased X-gal staining on the surface of the cerebellum' (Goodrich et al., 1997). These cells have been suggested to represent either a persistent external germinal layer or an early stage of tumorigenesis (Corcoran and Scott, 2001; Goodrich et al., 1997; Kim et al., 2003). The distinction is important: if these cells are essentially normal GCPs (except for their persistence into adulthood), their relevance for understanding tumorigenesis may be limited. However, if they are partially transformed and need only acquire a small number of additional changes to become tumors, then studying their properties may shed light on the earliest changes required in medulloblastoma. Isolation of these cells has allowed us to study their molecular and functional characteristics in detail. Although pre-neoplastic cells resemble GCPs in terms of location, lineage and hedgehog pathway activation, they differ significantly from GCPs in terms of gene expression, including loss of *patched*. In these respects, pre-neoplastic cells are much more similar to tumor cells. Based on these observations, it is likely that they represent an early stage of tumorigenesis.

In this context, it is worth noting that persistent EGL has been observed in other mutant mice. For example, animals with mutations in brain-derived neurotrophic factor (BDNF), matrix metalloproteinase 9 (MMP9), retinoid-related orphan receptor alpha (RORα/staggerer) or the peroxisome assembly gene PEX2 show delayed GCP migration and persistence of the EGL (Borghesani et al., 2002; Faust, 2003; Messer and Hatch,

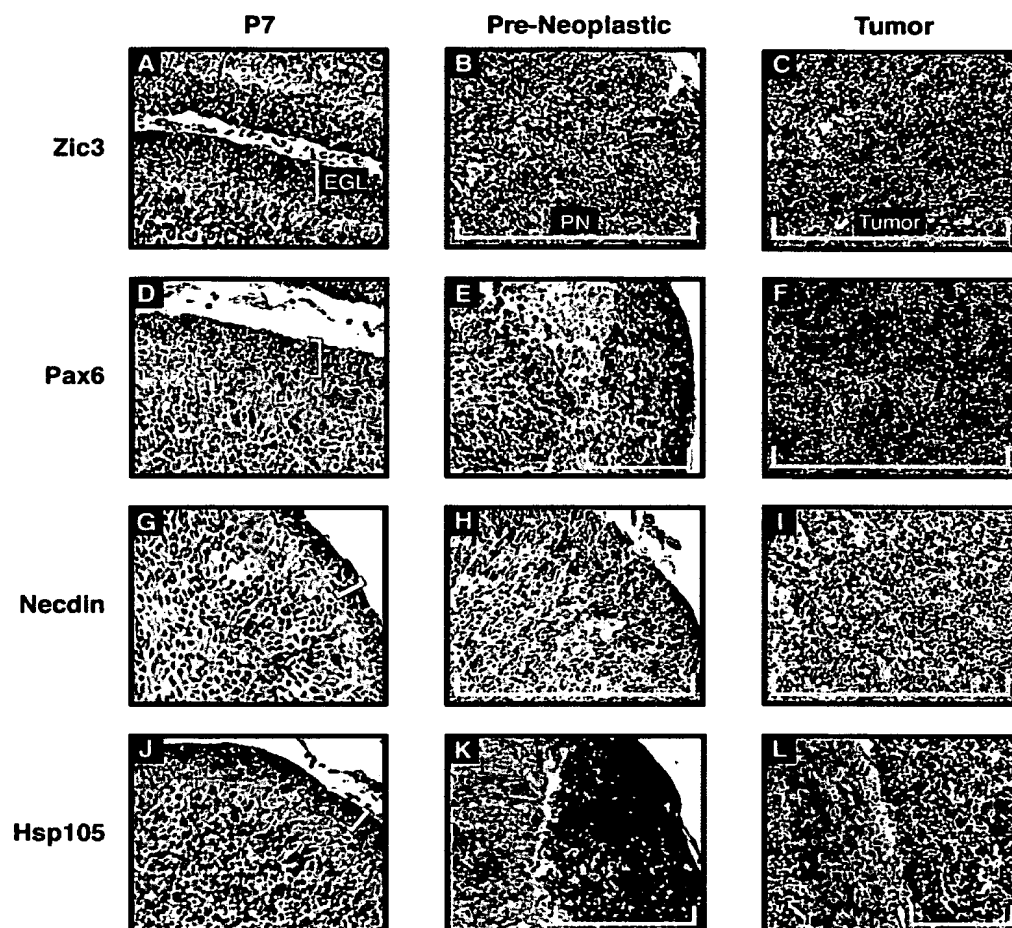


Fig. 10. Validation of microarray data by immunohistochemistry. Cryosections of neonatal (P7) cerebellum (A,D,G,J), pre-neoplastic lesions (B,E,H,K) and tumors (C,F,I,L) from *patched*^{+/-} mice were stained with primary antibodies specific for Zic3 (A-C), Pax6 (D-F), Necdin (G-I) or Hsp105 (J-L) and peroxidase-conjugated secondary antibodies. Staining was detected using the peroxidase substrate DAB, which yields a dark brown precipitate. Yellow brackets mark the boundaries of the EGL (panels A,D,G,J), pre-neoplastic (PN) regions (B,E,H,K) and tumor (C,F,I,L). Expression of all four proteins is detectable in GCPs within the EGL. Consistent with microarray data, expression of Zic3 decreases significantly in pre-neoplastic lesions and is absent from tumors; Pax6 and Necdin are restricted to peripheral regions at the pre-neoplastic stage and are undetectable in tumors; and Hsp105 expression increases markedly at the pre-neoplastic stage and decreases somewhat in tumors.

1984; Vaillant et al., 2003). Moreover, astrotactin 1 null mice exhibit ectopic accumulations of GCPs that superficially resemble the foci observed in *patched* heterozygotes (Adams et al., 2002). However, none of these animals develop medulloblastoma, suggesting that persistence of the EGL alone is not sufficient for tumorigenesis. The fact that the ectopic cells in *patched* mutant mice more closely resemble tumor cells than GCPs – and occur in animals that develop tumors – supports the use of the term ‘pre-neoplastic’ to describe these cells.

Pre-neoplastic cells arise from proliferating granule cell precursors

The fact that pre-neoplastic cells and tumor cells express markers of the granule cell lineage is consistent with the notion that they are derived from granule cell precursors. The same has been postulated for human medulloblastomas, particularly those with a desmoplastic appearance (Buhren et al., 2000; Kadin et al., 1970; Miyata et al., 1998). Desmoplastic tumors – which represent 20–30% of human medulloblastomas – frequently harbor mutations in the Shh pathway, and have a gene expression profile that resembles normal GCPs (Pomeroy et al., 2002). Interestingly, recent studies have demonstrated

that some human medulloblastomas (including desmoplastic tumors) express markers of neural stem cells (Hemmati et al., 2003; Singh et al., 2003; Singh et al., 2004). This could mean that they are derived from neural stem cells, or that they have acquired stem cell markers as a consequence of transformation (Oliver and Wechsler-Reya, 2004). Notably, few of the cells that we isolate from the *patched* mutant mice express markers of neural stem cells (nestin, CD133/prominin). Thus, tumorigenesis in the *patched* mutant mice does not appear to involve acquisition of a neural stem cell phenotype. Whether these cells exhibit other properties of neural stem cells – such as self-renewal or the capacity to differentiate into neurons and glia – remains to be determined.

Loss of *patched* and activation of the hedgehog pathway in pre-neoplastic cells

Our studies indicate that GCPs, pre-neoplastic cells and tumor cells express elevated levels of hedgehog target genes and proliferate in culture. In the case of GCPs, these responses probably reflect exposure to Shh in vivo shortly before isolation (Wechsler-Reya and Scott, 1999). In the case of pre-neoplastic cells and tumor cells, de-repression of the hedgehog pathway could result from loss of *patched* expression.

Although some studies have suggested that wild-type *patched* continues to be expressed in tumors (Romer et al., 2004; Wetmore et al., 2000; Zurawel et al., 2000), others have suggested that the wild-type *patched* locus in tumor cells may be silenced (Berman et al., 2002). Our studies support the latter view, demonstrating a striking loss of wild-type *patched* expression at both the pre-neoplastic and tumor stages.

One critical feature of our studies is the use of primers that distinguish between wild-type and mutant *patched* transcripts. In most studies that have reported continued *patched* expression in tumors from *patched* mutant mice, the data are based on northern analysis or in situ hybridization using probes that recognize both wild-type and mutant alleles. Although the mutant allele is clearly expressed in tumors, it is non-functional and would not be expected to contribute to the behavior of tumor cells. Our studies are also distinct in that we have separated pre-neoplastic and tumor cells from normal tissue. This is important because several cell types in the normal adult cerebellum express *patched* (Goodrich et al., 1997; Traiffort et al., 1999), and these cells can contribute significantly to RNA isolated from intact tumor tissue. By FACS-sorting cells that express Math1-GFP, we have eliminated these contaminating populations and analyzed expression of *patched* specifically in pre-neoplastic cells and tumor cells.

The mechanism by which *patched* is lost in pre-neoplastic cells and tumor cells remains unclear. Although studies of medulloblastoma cell lines derived from *patched*^{+/−}*p53*^{−/−} mice have demonstrated that the wild-type *patched* allele can be silenced by methylation (Berman et al., 2002), we have found no evidence for this in primary tumor cells from *patched*^{+/−} mice. Extensive sequencing of the four major CpG islands within and upstream of the *patched* promoter revealed no methylation in GCPs, pre-neoplastic cells or tumor cells (data not shown). Although methylation (or some other chromatin modification) could be present elsewhere in the *patched* gene, it is also possible that loss of *patched* expression results from mutations or deletions in the *patched* gene, or from loss of a signaling molecule or transcription factor that regulates *patched* expression. Mutational analysis of the *patched* locus, and studies of the transcription factors bound to the promoter, may help resolve this issue.

Regardless of the mechanism, our observation of decreased wild-type *patched* expression in both pre-neoplastic cells and tumor cells indicates that loss of *patched* is an early (and perhaps initiating) event in tumorigenesis. The fact that only a subset of pre-neoplastic cells develop into tumors implies that other changes besides loss of *patched* are required for the transition from the pre-neoplastic stage to more malignant stages of medulloblastoma. Whether these changes result from mutations or epigenetic events within pre-neoplastic cells themselves, or whether they arise from changes in the surrounding microenvironment, is an important question for future study.

Identification of genes associated with medulloblastoma progression

GCPs, pre-neoplastic cells and tumor cells resemble one another in many ways. And yet, GCPs are present in all of *patched* mutant mice (at the neonatal stage), pre-neoplastic cells are present in over half of these animals (at 6 weeks), and

tumors occur in only 15–20%. Thus, these populations must differ from one another. To identify intrinsic differences between GCPs, pre-neoplastic cells and tumor cells, we analyzed gene expression using microarrays. These studies revealed that the three populations are remarkably similar when compared with normal adult cerebellum, but show significant differences when compared directly to one another. (Notably, because all three populations exhibit hedgehog pathway activation and proliferation, differentially expressed genes include few hedgehog targets or components of the cell cycle machinery. Rather, major differences are detected in genes associated with cell migration, survival and differentiation.)

Our approach differs from several recent studies of gene expression analysis in medulloblastoma (Boon et al., 2003; Kho et al., 2004; Lee et al., 2003; MacDonald et al., 2001; Park et al., 2003; Pomeroy et al., 2002; Wechsler-Reya, 2003). In most cases, these investigators compared medulloblastoma with other brain tumors or with normal adult cerebellum. For example, Pomeroy et al. found that medulloblastoma has a distinct gene expression profile compared with other pediatric brain tumors (Pomeroy et al., 2002). In particular, desmoplastic medulloblastomas (which often harbor mutations in the hedgehog pathway) have elevated expression of hedgehog target genes such as *Nmyc*, *patched*, *gli1* and *IGF2* (insulin-like growth factor 2). Similarly, Boon et al. used serial analysis of gene expression (SAGE) to compare human medulloblastoma and normal brain, and found increased expression of hedgehog targets and cell cycle regulators such as *Nmyc* and thymidylate synthase (Boon et al., 2003; Oliver et al., 2003). These findings are consistent with the fact that medulloblastoma cells are highly proliferative cells that exhibit hedgehog pathway activation, whereas normal brain consists primarily of post-mitotic neurons in which the hedgehog pathway is inactive. In contrast to these studies, we compared medulloblastoma cells with their cells of origin, proliferating GCPs. This allowed us to control for proliferation and hedgehog pathway activation and, instead, to identify other genes that may play an important role in tumor progression.

Similar to our studies, Lee et al. analyzed gene expression in medulloblastoma and in neonatal cerebellum (Lee et al., 2003). Both studies found that gene expression in medulloblastoma was much more similar to neonatal cerebellum than to adult cerebellum. Yet there were also key differences between these studies. In particular, Lee et al. found that medulloblastoma is characterized by increased expression of genes associated with the granule cell lineage, hedgehog pathway activation and proliferation (*math1*, *srebp*, *hexokinase*, *Nmyc*, *cyclin D1*, *sfrp1*), whereas we did not detect elevated expression of these genes. One important distinction between the two studies is the use of intact tissues versus dissociated cells. Lee et al. compared intact tumor tissue (which consists largely of GCP-like cells) and intact neonatal cerebellum (which contains not only GCPs but also significant numbers of post-mitotic granule cells and other cell types). By contrast, we compared purified populations of GCPs, pre-neoplastic cells and tumor cells, all of which are highly enriched for proliferating GCP-like cells. As a result, genes associated with cell lineage and degree of hedgehog pathway activity were not differentially expressed in our screen.

Dysregulation of migration, differentiation and apoptosis in medulloblastoma

Our study is unique in that we compared gene expression in similar populations of cells at three different stages of tumor progression. This allowed us to identify genes that distinguish these stages. Interestingly, many of these genes were regulators of migration, survival and differentiation, processes that have been studied in the context of normal granule cell development but were not previously known to play a role in medulloblastoma. Most prominent among these genes were regulators of cell migration. These included transcription factors (Pax6), surface receptors (Unc5h3), secreted proteins (PAF acetylhydrolase) and ECM molecules (Collagen) that have been implicated in granule cell migration (Engelkamp et al., 1999; Fishman and Hatten, 1993; Przyborski et al., 1998; Tokuoka et al., 2003). Notably, the majority of the genes we identified have been found to promote migration, and were downregulated in pre-neoplastic cells. Decreased expression of these genes during the early stages of tumorigenesis is interesting because pre-neoplastic cells clearly exhibit aberrant migration: whereas GCPs migrate inward during normal development, pre-neoplastic cells (and tumor cells) remain stuck on the surface of the cerebellum. A similar location has been noted for human medulloblastomas, which often spread through the meninges but rarely invade the inner layers of the cerebellum (Koeller and Rushing, 2003).

It has long been unclear how failure to migrate inward is related to tumorigenesis. One possibility is that pre-neoplastic cells and tumor cells are unable to migrate because they are proliferating or locked in a precursor state. Alternatively, the inability to migrate could be an intrinsic defect that plays an essential role in tumorigenesis. For example, by losing the ability to migrate, cells may remain trapped in an environment (e.g. close to the pial surface) that facilitates their continued growth and survival. Our finding that genes involved in migration are downregulated in pre-neoplastic cells strongly supports the notion of an intrinsic defect. However, further experiments will be necessary to determine whether changes in migration-associated genes are necessary for the development of medulloblastoma.

Genes that regulate differentiation may also be crucial in determining the course of tumorigenesis. Importantly, we found that pre-neoplastic cells have reduced expression of genes associated with granule cell differentiation (e.g. *Zic3* and *Neurod1*). This is consistent with the fact that these cells continue to proliferate and retain expression of GCP markers, whereas normal GCPs can exit the cell cycle and differentiate into mature granule neurons. Whether the inability to differentiate is a cause or a consequence of transformation remains to be determined, but the identification of specific transcription factors that may regulate this process will allow us to test the significance of differentiation directly. Further studies perturbing the expression of these factors in vivo are underway.

Finally, our data suggest that altered stress responses may contribute to tumor formation in *patched* mutant mice. Heat-shock proteins, co-chaperones (*Hsp60*, *Hsp105*, *Dnajb1*, *Dnajb10* and *Bag3*) and the transcription factor *Atf3* all function to protect cells from stress-induced apoptosis and necrosis (Hatayama et al., 2001; Nakagomi et al., 2003;

Takayama et al., 2003). The increased expression of these genes in pre-neoplastic cells and tumor cells may reflect an increased ability to survive under stress conditions, which may be crucial for the early stages of tumorigenesis. In fact, several lines of evidence suggest that dysregulation of apoptosis or stress responses may be important for medulloblastoma formation. For example, crossing *patched* mutant mice with homozygous *p53* knockout mice leads to a dramatic increase in tumor incidence: whereas only 14–20% of *patched* heterozygotes develop medulloblastoma, more than 95% of *patched*^{+/-}/*p53*^{-/-} mice develop tumors (Wetmore et al., 2001). Similarly, animals that lack both *p53* and *PARP1*, an enzyme that promotes cell death in response to DNA damage, develop medulloblastoma (Tong et al., 2003). Notably, no mutations in *p53* have been found in tumors from *patched* heterozygotes (Wetmore et al., 2001), suggesting that in these animals some other element of the apoptotic machinery may need to be inactivated for tumors to form. The genes we have found to be upregulated in pre-neoplastic cells may provide some important clues to the mechanisms by which apoptosis is subverted in medulloblastoma.

The identification of a pre-neoplastic stage in murine medulloblastoma raises the possibility that a similar stage exists in the human disease. If so, the genes we have identified as markers of pre-neoplastic cells may be useful for early detection of medulloblastoma, particularly in people with an inherited susceptibility to the disease (Gorlin, 1987; Hamilton et al., 1995). Detection of medulloblastoma at early stages may increase the effectiveness of conventional medulloblastoma therapy. In addition, if the genes we have identified play a causal role in tumor progression, they may serve as valuable targets for therapy. Recent studies suggest that pharmacologic antagonists of the hedgehog pathway may be effective at inhibiting growth of medulloblastoma cells in vitro and in vivo (Berman et al., 2002; Romer et al., 2004). Therefore, identification of other pathways that are crucial for tumorigenesis may open up new avenues for treatment of this devastating disease.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/10/2425/DC1>

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REVIEW ARTICLE

Apoptosis and the Dilemma of Cancer Chemotherapy

By Yusuf A. Hannun

IT WAS 25 YEARS AGO that the war against cancer was officially launched by President Nixon. The ensuing investment in the study of cancer has been a major engine driving the current revolution in cell and molecular biology. Yet, the clinical rewards have been less than remarkable. This runs the danger of raising the level of skepticism among the public and may erode the level of support, just when it is needed most to effect a translation of the accomplishments of basic research into the fruits of clinical medicine.

Over the last 3 to 5 decades, the treatment of cancer has relied primarily on the use of various forms of cytotoxic chemotherapy and radiation therapy. These interventions have had profound positive results on many hematologic malignancies and a few solid tumors, especially germ cell and some childhood malignancies. However, the most prevalent of malignancies have proved to be more or less resistant to these interventions. Dose escalation using high-dose chemotherapy may have resulted in a modest improvement in responses but has not constituted a breakthrough. In all these cases, the effectiveness of cytotoxic treatments has been limited by the side effects of these agents on normal tissues and cells, even with the successful attempts of reducing these toxicities using various forms of supportive measures (such as bone marrow rescue, antibiotics, antiemetics, and growth factor support).

On the other hand, recent accomplishments in the understanding of mechanisms of growth regulation, stress response, and the action of several cytotoxic agents may allow us to begin to develop a conceptual framework for pursuing cancer chemotherapy at the cell and molecular level. This may then translate into a goal-directed and rational approach to cancer treatment.

Indeed, accruing evidence obtained in the last few years is beginning to establish that many (and perhaps all) agents of cancer chemotherapy effect tumor cell killing in vitro and in vivo through launching the mechanisms of apoptosis (or programmed cell death). These lines of investigation have provided significant insight into the mechanisms involved in cell death in general and tumor cell death more specifically. Such insight may allow the identification of novel targets and the development of more specific chemotherapeutic agents that are designed to launch specifically the apoptotic machinery of the cell. Paradoxically, the realization that chemotherapeutic agents (and possibly ionizing irradiation) are

effective primarily because they activate apoptosis raises the concern that tumors that are intrinsically resistant to chemotherapy are unable to activate the apoptotic machinery and may therefore be fundamentally resistant to chemotherapeutic cell death.

This review will highlight the recent developments in the field of apoptosis in general (especially at the mechanistic level), provide evidence linking chemotherapy-induced cell death and apoptosis, formulate a hypothesis for cancer chemotherapy, and conclude with a discussion of the ramifications of such a formulation for the future development of cancer chemotherapy.

APOPTOSIS: DEFINITIONS AND MECHANISMS

The History and Biology of Apoptosis

Apoptosis has become one of the hottest areas of cell biology research, probably because of the belated realization that cell death is a biochemically regulated process that may be as complex as other fundamental biological processes. The existence of various forms of cell death involving tissues and cells was recognized in the 19th century, although it never received primary attention.¹ Programmed forms of cell death have also been recognized in the field of botany, but it has been labeled mostly as senescence. In 1970, Wyllie and Kerr formalized the existence of a human form of cell death distinct from necrosis that they termed apoptosis.^{2,3} Apoptosis received its primary boost with the identification of internucleosomal DNA breakdown during apoptosis and not necrosis.² Because this form of DNA breakdown suggested the action of an endonuclease, this singular finding

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may have convinced many investigators that apoptosis is the manifestation or outcome of biochemical processes.

Apoptosis is now recognized as a mechanistically driven form of cell death that is either developmentally regulated,³ launched in response to specific stimuli (such as the cytokines tumor necrosis factor α or the fas ligand),^{4,5} or activated in response to various forms of cell injury or stress.^{6,7} In developmental biology, programmed cell death is responsible for eliminating superfluous or redundant precursor or mature cells. For example, in immunobiology, apoptosis accounts for the elimination of self-reacting lymphocytes.⁸ Apoptosis also appears to play an important role in tissue remodeling and reaction to the environment whereby unnecessary cells may undergo cell death to allow the growth and differentiation of cells that are better geared to deal with the changing environmental demands.³ In cancer biology, it is becoming increasingly apparent that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self-destruction, which would have been indicated because of the many mutations they may harbor.^{9,10} Indeed, disarming apoptosis and other surveillance mechanisms may be of fundamental significance in allowing the development of the malignant and metastatic phenotype.

Mechanisms of Apoptosis

In the 1980s, two major endogenous regulators of apoptosis were identified. Although initially identified as an oncoprotein, it soon became clear that the wild-type p53 protein functioned as an inducer of cell death, especially in response to DNA damaging events.¹¹⁻¹³ Reciprocally, studies on the Bcl-2 oncogene led to the identification of an important antiapoptotic function for this protein that therefore gave survival advantage to lymphomas that overexpressed the protein product.^{14,15} Bcl-2 is now appreciated to belong to a family of related and interacting molecules such as Bax, Bcl-x, Bad, Bag, Bak, and Bik, some of which are antiapoptotic, whereas other members of the family, such as Bax, display pro-apoptotic function.¹⁵⁻¹⁷

In independent lines of investigation, investigators determined the existence of specific genes that regulate cell death during the development of *Caenorhabditis elegans*, including a homologue of Bcl-2 and a protease that is a member of the interleukin-converting enzyme (ICE) family of cysteine proteases.^{18,19} These key developments appear to have convinced the scientific community that apoptosis and cell death are regulated outcomes. As a consequence, an intensive effort has been applied in the recent past aimed at defining mechanisms involved in the initiation and execution of cell death. These intensive efforts have resulted in a flurry of activity and a wealth of information as well as the realization that this is a complex process with many as yet undiscovered components and processes that ultimately regulate apoptosis. There are numerous reviews^{2,3,6-10,14,15,20-28} that cover various aspects of apoptosis, its historical development, components involved in apoptosis, and mechanisms regulating apoptosis.

Mechanisms regulating cell death are perhaps best exemplified in cytokine-induced apoptosis (Fig 1). For example, both tumor necrosis factor α and the Fas ligand cause the

death of certain malignant and normal cells. Activation of the receptors for these cytokines launches an intracellular pathway through specific proteins^{29,30} that interact with these receptors (FADD, TRADD, and others). These proteins appear to couple to a protease (MACH/FLICE), and activation of this protease propagates the apoptotic signal.^{31,32} A component of this pathway includes sphingomyelinases that act on membrane sphingomyelin and cause the release of the lipid mediator ceramide.^{33,34} Ceramide may then cause the activation of downstream proteases that, in turn, seem to launch the final phase of apoptosis. This phase involves the breakdown of several macromolecules including DNA and proteins, fragmentation of organelles, and packaging of the cellular debris into apoptotic bodies that are then engulfed by phagocytic cells. Through as yet unidentified mechanisms, Bcl-2 appears to interfere with the activation of the distal proteases,^{35,36} whereas p53 seems to launch a more proximal component of this pathway. Obviously, major gaps exist in connecting the different components of this scheme and in defining the relative contribution of these components. Nevertheless, this is a very promising beginning towards unraveling the regulation of this important process.

CANCER CHEMOTHERAPY AND APOPTOSIS

Chemotherapeutic Agents Kill Susceptible Cells by Apoptosis

In a landmark study investigating the mechanism of action of etoposide (an inhibitor of topoisomerase II) and other chemotherapeutic agents, it was found that etoposide, early on, induced internucleosomal DNA fragmentation.³⁷ This observation raised the possibility that etoposide caused apoptotic cell death. Since then, the spectrum of chemotherapeutic agents causing apoptosis has expanded progressively, and the evidence supporting the role of apoptosis in chemotherapy action continues to accumulate. The chemotherapeutic agents that have thus far been identified as apoptosis-inducing include etoposide, VM26, m-AMSA, dexamethasone, vincristine, cis-platinum, cyclophosphamide, paclitaxel, 5'-fluoro-deoxyuridine, 5'-fluorouracil, and adriamycin³⁷⁻⁴¹ (Table 1). These apoptotic effects have been observed in several cell lines in tissue culture, including normal thymocytes, lymphoma cells, ovarian epithelial tumors, leukemia cells, adenocarcinoma cells, and others. In addition, tumor hypoxia, ionizing radiation, and hormone withdrawal in hormone-dependent tumors have been shown to cause apoptosis.⁴²⁻⁴⁷ The occurrence of apoptosis has been documented by morphologic criteria, the occurrence of endonucleosomal DNA breakdown, flow cytometric analysis of DNA content, and other criteria. The reader is referred to multiple reviews that document the ability of chemotherapeutic interventions to cause apoptosis.^{9,14,48-50}

Other studies are also beginning to provide evidence that chemotherapeutic agents induce apoptotic tumor cell death in vivo. For example, a retinoic acid-treated T-cell lymphoma was shown to undergo apoptosis in vivo.⁵¹ In a study of esophageal squamous cell carcinoma, it was shown that both radiation and chemotherapy (5-fluorouracil, cis-

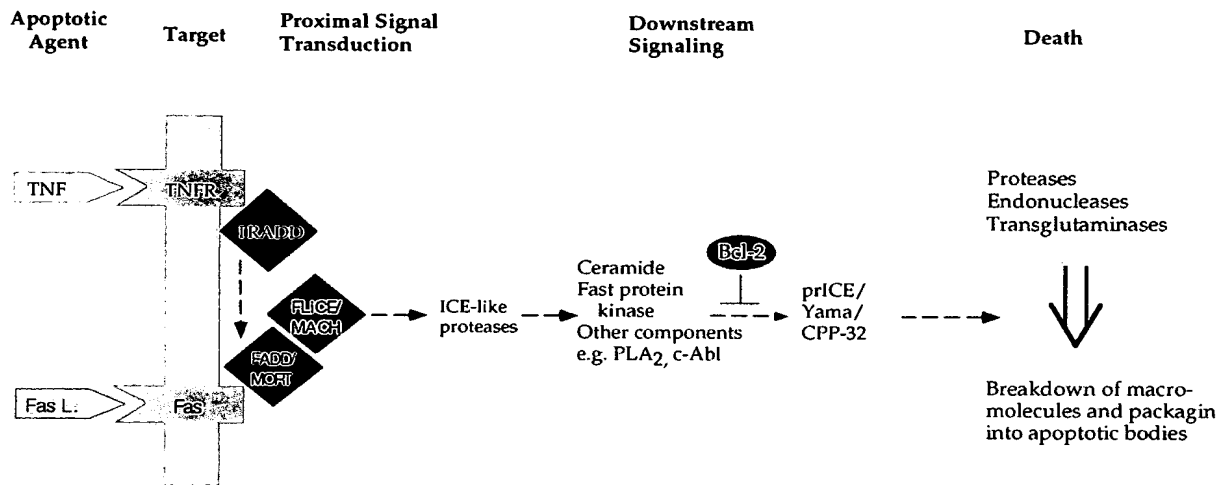


Fig 1. Scheme for induction of apoptosis in response to the cytokines tumor necrosis factor α and the Fas (APO-1) ligand. Both cytokines bind to specific membrane receptors (such as TNFR and Fas) and allow for the interaction of the receptors with specific proteins (such as TRADD and FADD/MORT). This may result in activation of a protease (Mach/Flice) that somehow launches the apoptotic response. Downstream mediators of this response may include sphingomyelinase causing the accumulation of ceramide, phospholipase A2 with accumulation of arachidonate, and other mediators such as the Fast kinase.²⁴ Eventually, this results in activation of downstream proteases such as prICE/CPP-32, which cleaves PARP and possibly other protein substrates. This then appears to induce the terminal and irreversible phase of apoptosis characterized by macromolecular breakdown and cellular fragmentation.

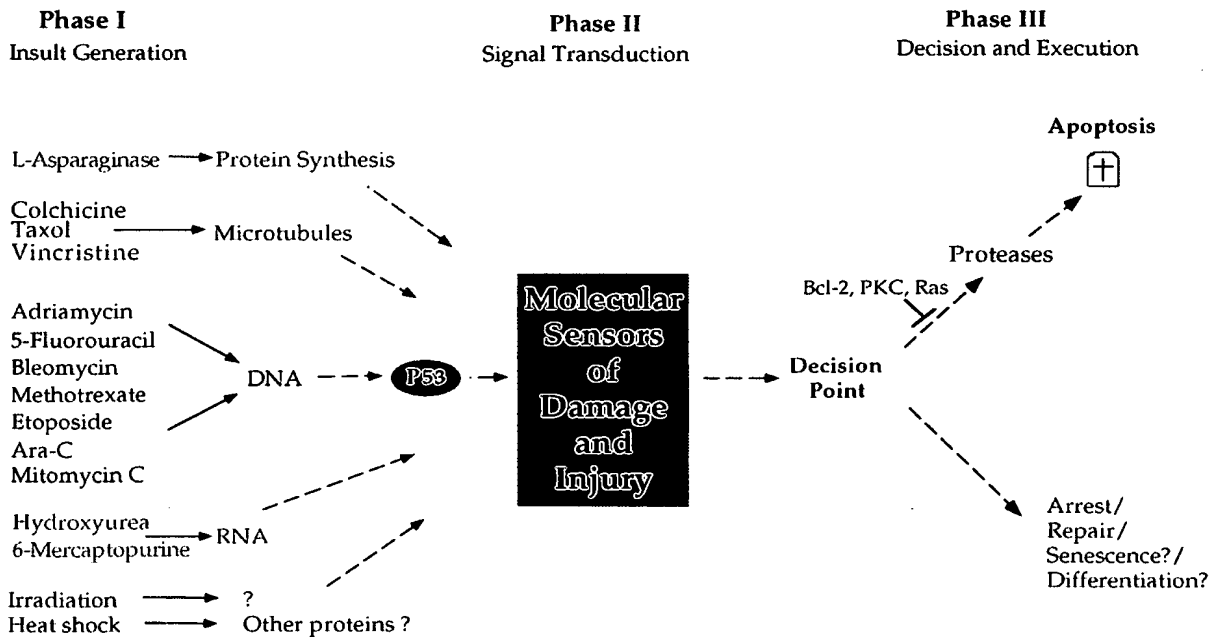


Fig 2. Hypothesized phases in the induction of apoptosis in response to chemotherapeutic agents. In phase I, cytotoxic agents impart damage to a critical component of the cell such as DNA or microtubules. In phase II, the cell recognizes the damage and its degree of severity through poorly characterized signaling mechanisms. In phase III, the cell assesses the extent of damage and decides on the appropriate response. In many cancer cells, the preferred response is the induction of apoptosis, whereas in most normal cells and in many cancer cells, the response may involve growth arrest to allow for repair. It is also possible that certain cells may react to damage by undergoing senescence or terminal cell differentiation. Cancer cells may acquire resistance to apoptosis at several points in this pathway. For example, mutant p53 may impart resistance to DNA-damaging agents; mutations may exist in the signaling phase (phase II) or in the apoptotic phase III such as with mutant Bcl-2, mutant ras, or hyperactive protein kinase C (PKC).

platinum, and bleomycin) induced apoptotic cell death in vivo, as determined by examination of biopsy specimens.⁵² In an experimental study in murine tumors, evidence was also provided that cis-platinum, cyclophosphamide, and other chemotherapeutic agents caused apoptosis in several in vivo tumors, including adenocarcinoma, lymphoma, sarcomas, and squamous cell carcinomas.⁵³ (Although not all agents caused apoptosis in all the tumors.) Similarly, in a study of murine mammary adenocarcinoma and ovarian carcinoma, it was observed that cyclophosphamide treatment increased apoptosis in these tumors.⁵⁴ Antileukemic therapy (including etoposide, m-AMSA, and cytosine arabinoside) caused apoptotic cell death in patients undergoing chemotherapy for acute leukemia.⁵⁵

Mechanisms of Chemotherapy-Induced Apoptosis

With the developing understanding of mechanisms regulating apoptosis, it is becoming increasingly clear that chemotherapeutic agents operate through similar mechanisms. Indeed, some of the insight into mechanisms regulating apoptosis has come from the examination of chemotherapy-induced death. This is best illustrated again with the case of etoposide in which Kaufmann et al.⁵⁶ identified proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) in response to etoposide. Subsequent studies indicated that this was a result of activation of a specific protease and that it preceded endonuclease activation and DNA fragmentation.⁵⁷ This PARP protease has evolved as a centerpiece in the study of apoptotic mechanisms, and it has been cloned by several investigators and has been given several names, including CPP-32, pICE, Yama, and apopain.⁵⁸⁻⁶⁰ It now appears that many inducers of cell death, including cytokines and other chemotherapeutic agents, ultimately converge on the activation of this and related proteases, which then appear to launch the terminal and execution stages of apoptosis (Fig 1).

Another convincing set of data supporting the role of apoptosis in chemotherapy action has come from studies on the interaction of chemotherapeutic agents with modulators

of apoptosis. Bcl-2 overexpression has been shown to inhibit apoptosis in vitro in response to several chemotherapeutic agents, including etoposide, methylmethanesulfonate, N'-methyl-N'-nitrosourea, dexamethasone, camptothecin, doxorubicin, 4-hydroxyperoxy-cyclophosphamide, vincristine, and actinomycin D.^{14,61-67} In addition, the level of expression of Bcl-2 in clinical tissue samples has been correlated with disease prognosis in non-Hodgkin's lymphoma,⁶⁸ but not in childhood acute lymphoblastic leukemia.⁶⁹ On the other hand, reduced expression of Bax, a pro-apoptotic homologue of Bcl-2, was found to be associated with poor response to combination chemotherapy and worse survival in patients with metastatic breast carcinoma.⁷⁰ In addition, many chemotherapeutic agents, such as cytosine arabinoside, vincristine, daunorubicin, and ionizing radiation, have been shown to cause accumulation of ceramide.⁷¹⁻⁷⁴ In the case of ionizing radiation, it has been shown recently that this may involve activation of an acidic sphingomyelinase and that mice lacking this enzyme acquire resistance to radiation-induced apoptosis in the lung.⁷⁵

Other modulators of apoptosis have also been shown to interact with chemotherapy-induced cell death. For example, the cytotoxicity of several chemotherapeutic agents, including vincristine, adriamycin, cytosine arabinoside, and cyclophosphamide, has been shown to be inhibited by hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, or interleukin-6.⁷⁶ Disruption of p53 protects breast carcinoma cells from platinum-induced death,^{77,78} and BCR-ABL protects from apoptosis induced by a variety of chemotherapeutic agents.⁷⁹ The Epstein-Barr viral protein BHRF1, which shows structural⁸⁰ and functional⁸¹ homology to Bcl-2, was shown to protect against cell death induced by etoposide and cisplatin,⁸² and the expression of bcl-x was shown to modulate drug sensitivity of breast cancer neuroblastoma^{83,84} and prolymphocytic⁸⁵ cells. The oncogenes H-ras and MDM2 were shown to confer resistance to chemotherapy-induced apoptosis in rhabdomyosarcoma and glioblastoma, respectively.^{86,87} Safingol, an inhibitor of protein kinase C (which is usually associated with a viability response), was found to potentiate the ability of Mitomycin C to kill gastric cancer cells.⁸⁸

These in vitro and in vivo studies, coupled with the mechanistic insight evolving on the regulation of chemotherapy-induced cell death, clearly show the induction of apoptosis by several chemotherapeutic agents in different cell lines and tumors. On the other hand, it has not yet been ascertained what fraction of tumor cell death is effected through apoptotic mechanisms. Determining this fraction may not be trivial, but it could underscore the extent of apoptosis and its significance in chemotherapy-induced death. (It is anticipated that the more we learn about chemotherapy-induced cell death, the more we will appreciate the extent and significance of apoptosis in mediating chemotherapy-induced cytotoxicity.)

HYPOTHESIS AND RAMIFICATIONS

If, indeed, various agents of cancer chemotherapy act primarily through the induction of apoptotic cell death in sus-

Table 1. Cancer Therapy and Apoptosis

Some Agents Associated With Apoptosis		Cancer Types Susceptible to Apoptosis
Etoposide	Camptothecin	Lymphoma
VM26	Hydroxyurea	Leukemia
m-AMSA	Ara-C	Breast carcinoma
Dexamethasone	5-Azacytidine	Ovarian carcinoma
Vincristine	Nitrogen Mustard	Colon carcinoma
cis-Platinum	Methotrexate	Prostate cancer
Cyclophosphamide	Chlorambucil	Miscellaneous
Adriamycin	Bleomycin	adenocarcinomas
Paclitaxel	BCNU	Seminoma
5'-Fluorouracil	Actinomycin D	Malignant glioma
5'-Fluoro-deoxyuridine	Melphalan	
Ionizing radiation		
Hyperthermia		
Hormone withdrawal		

ceptible cancer cells, then how can we reconcile the existence of distinct targets for these disparate chemotherapeutic agents with the more or less unifying concept of apoptosis?

Hypothesis

The simplest, yet most encompassing, hypothesis (Fig 2) proposes that each chemotherapeutic agent interacts with a specific target causing dysfunction and injury, which is then interpreted by susceptible cancer cells as an instruction to undergo apoptosis. Thus, we may consider that chemotherapy-induced cell death proceeds through three distinct general phases. (1) Phase I: an insult-generating mechanism. In this phase, each class of chemotherapeutic agents interacts with a specific target such as DNA, RNA, and microtubules and the action of these agents on their respective targets causes target injury or dysfunction. (2) Phase II: signal transduction. In this phase, the cell, through yet poorly defined mechanisms, is able to decipher and assess the specific injury to the chemotherapy target. For example, DNA-damaging agents may use the c-Abl tyrosine kinase to induce cell cycle arrest in a p53-dependent mechanism.⁸⁹ Treatment of some T-cell leukemia lines with doxorubicin results in upregulation of the ligand to the Fas receptor, which may mediate the ability of doxorubicin to kill these cells.⁹⁰ Such a mechanism may account for the sensitivity of leukemia cells to chemotherapy. As described in the previous section, the existence of such mechanisms for transducing cell death in response to cytokines is becoming increasingly appreciated and defined, and further studies should clarify the mechanisms involved in transducing the apoptotic responses to chemotherapeutic agents. (3) Phase III: induction of apoptosis. In the third and final phase, a decision point may exist such that susceptible cells react to the signals generated in response to chemotherapy-induced injury as a go-ahead for the execution phase of apoptosis. For example, γ -radiation causes the induction of the pro-apoptotic Bax in radio-sensitive but not in radio-resistant cells. This may drive cells to apoptosis.⁹¹ In this phase, the cells undergo the orderly breakdown of macromolecules through the operation of proteases, endonucleases, transglutaminases, and possibly lipases. Other cell types may preferentially execute programs of cell cycle arrest and damage repair in response to these same signals. Although such a decision point appears critical in imparting selectivity of responses to similar stimuli, little is known concerning its existence, components, or regulation.

Analogy: The Cell as a Highly Organized Factory Owned by a Large Multinational Company

A useful analogy would be to think of the cell as a highly efficient production facility with different components charged with specific functions (such as engines, batteries, software, etc). For the sake of high efficiency, the activities of these different components must be highly coordinated so that the final product is not seriously limited by any one component. This also ensures that energy is not wasted on overproduction of other components. This necessitates that

the function and status of each component be monitored closely and that these monitoring systems interact and feed back into each other. According to this analogy, if one component receives an insult (such as a fire affecting the battery-producing facility), then the factory has to make a decision as to whether to slow down other production facilities to allow repair (analogous to cells undergoing cell cycle arrest to take care of DNA damage). If the injury is severe and leads to irreversible damage (complete burn down), then the decision may be to permanently close down the production facility and move viable functions to other existing and better-suited production units so as to minimize the losses of the mother company (cells altruistically undergoing irreversible apoptosis for the sake of the tissue or organism).

Ramifications

This formulation suggests a number of ramifications that ultimately impact not only on how we perceive of cancer chemotherapy, but also, more importantly, on what we need to do to achieve a new level of success in cancer therapeutics.

New targets for chemotherapy. As discussed above, most chemotherapeutic agents in current practice appear to induce damage to a major component in the cell, ie, they appear to act in phase I of the proposed scheme (Fig 2). The first ramification suggested by the apoptosis hypothesis stipulates that cancer cell death may be effected by bypassing the initial targets of currently existing chemotherapeutic agents. That is, agents could be developed that either target phase II (ie, falsely signaling damage) or phase III (instructing the cell to undergo apoptosis directly). An advantage to this approach is that it may bypass many of the current hurdles and obstacles facing chemotherapeutic agents. This may be considered the mechanistic approach to cancer chemotherapy. Its development requires significantly more understanding of the various components of phases II and III and how they can be modulated therapeutically.

Why are some cancer cells prone to apoptosis? This component of the hypothesis suggests that most normal cells in the human body are intrinsically much more resistant to apoptosis than several cancer types. Indeed, some experimental evidence shows that diploid fibroblasts are more resistant to apoptosis and actually undergo cell cycle arrest in response to many of the agents that would cause leukemia and lymphoma cells to undergo apoptosis (M. Smyth and Y. Hannun, unpublished observations). This is also supported by the observation that activation of p53 fails to induce apoptosis in normal diploid fibroblasts; however, transformation of these cells renders them susceptible to apoptosis.⁹² Why are then some cancer cells more susceptible to apoptosis? Unfortunately, at this point in time very little is known concerning the mechanisms that determine whether a cell undergoes apoptosis, cell cycle arrest, or other responses to agents of injury. Understanding the biochemical and molecular bases for this decision making by the cell (Fig 2) could allow a better therapeutic window to drive more cancer cells into apoptosis while preserving normal cells. This would result in better selectivity for cancer chemotherapy.

It should also be noted that conceptualizing the rationale for the increased susceptibility of cancer cells to apoptosis is somewhat counterintuitive. Current understanding of molecular cancer pathogenesis suggests that cancers should be more resistant to apoptosis as a mechanism to escape self-elimination. This problem is revisited in the next section.

Why are most cancers resistant to apoptosis? The third ramification of this hypothesis concerns the issue of resistance. Why are certain tumor cells more intrinsically resistant to apoptosis? Are they more resistant than normal cells? And what can we do about it? The major contention of this hypothesis is that most solid tumors are intrinsically resistant to apoptosis in general and to chemotherapy-induced apoptosis in particular. These cancers may very well have disarmed regulatory mechanisms that survey damage or injury. This is supported by the frequent mutations in p53, which appears to play a critical role in the response to DNA damage. Other mutations may exist that also allow these cancer cells to escape suicide. If chemotherapeutic agents act primarily to induce apoptosis, then it becomes obvious that these cancer cells may be a priori resistant to cell death in response to these agents. If this is the case, then the hematologic and germ cell malignancies may be the exceptional malignancies. For some reason these cancers may have traded the increased susceptibility to apoptosis for some survival or growth advantage. What the era of cytotoxic chemotherapy may have accomplished is to segregate cancers into two groups: those that are prone to apoptosis and those that are resistant!

At this point in time it is not known if the resistant cancers have defects solely in phase II or also defects in phase III. By having defects in either phase II or III, these cells would be very resistant to any agent that acts in phase I. Therefore, pursuing classical cytotoxic chemotherapy would be a futile endeavor.

THE DILEMMA

Addressing these issues of resistance could either provide us with a rational approach to cancer therapy or it could present us with (the insurmountable dilemma: many cancer cells are at least as intrinsically resistant to apoptosis as normal cells. There are no windows of opportunity to selectively kill cancer cells without killing the host tissues!)

Let us consider a few possibilities as to what could make cancer cells resistant to chemotherapy (Fig 2).

(1) A cancer cell has a defect in one arm of induction of apoptosis, such as a mutation in p53, and the cell becomes resistant to activators of that arm (such as DNA-damaging agents). All other components of the apoptotic pathways are intact. This resistance could be overcome by using agents that either act on another arm in phase I or bypass the defect by acting further downstream. This is probably not a very common scenario because (the cancers we need to deal with most are the ones that appear to be resistant to a variety of apoptosis-inducing agents.)

(2) A cancer cell has one or more mutations or defects in the common signaling components of phase II, but otherwise the downstream apoptotic machinery is intact. This resis-

tance can be overcome by developing agents that act later in phase II or in phase III. This would result in therapy based on apoptosis only.

(3) A cancer has a mutation or a change in a gene that provides a selective survival advantage (eg, overexpression of Bcl-2). If these blocks are undone or bypassed (eg, agents that downregulate or act downstream of Bcl-2), then the cancer cells can be killed. Indeed, antisense approaches to Bcl-2 (which attenuate the expression of Bcl-2) have been shown to relieve the block to apoptosis in leukemia cells with elevated expression of Bcl-2.⁹³ Mutations in ras, which may confer resistance to chemotherapy,⁸⁶ may be overcome by specific approaches that target the function of ras (such as inhibitors of prenylation of ras). Such approaches may result in combination chemotherapy with a cytotoxic agent coupled to a specific agent designed mechanistically for the particular cancer.

(4) The cancer cells have either a defect in the apoptotic machinery or are unable to respond to damage by undergoing apoptosis. The existence of such a defect may allow the selective killing of cancer cells if the defect is repaired or overcome. On the other hand, (these cells may resemble normal cells in their intrinsic resistance to apoptosis: their decision process may be more similar to normal cells in that they may preferentially undergo cell cycle arrest or other nonapoptotic responses to damaging insults. In this case, selectivity over normal cells may not be achievable.) Alternative strategies have to target treatments to the cancer cells specifically to avoid toxicity to normal cells (eg, antibody- or receptor-based targeting). A recent and promising example is the use of an engineered adenovirus that selectively replicates in and kills cells lacking a functional p53.⁹⁵

UNANSWERED QUESTIONS

There is an emerging realization that cancer chemotherapeutic agents act primarily by inducing cancer cell death through the mechanisms of apoptosis. This review has attempted to provide the foundation for this concept and develop it further with the aim of generating the next level of hypotheses and questions: (1) Does apoptosis account for all, or at least most, of the action of chemotherapeutic agents? (2) What are the intracellular mechanisms responsible for transducing the apoptotic signals generated by chemotherapeutic agents? (3) What are the general mechanisms operating in the induction of apoptosis (the mechanisms that operate with cytokines, injury, stress, irradiation, starvation, etc)? (4) Can we target directly the machinery of apoptosis in cancer cells and effect cancer cell death? (5) What makes some cancers (primarily leukemias and lymphomas) very prone to apoptosis? Is this peculiar to these malignancies or does it extend to other curable cancers? Can we use this knowledge to render resistant cancers sensitive? (6) What are the mechanisms that bestow on most solid tumors resistance to chemotherapy? Is it because of a generalized resistance to apoptosis? Can this be bypassed? (7) Can we find experimental windows of opportunity that allow us to induce cancer cell death selectively without killing normal cells?

These questions and hypotheses should provide a founda-

tion for a rational approach towards cancer chemotherapy based on the premise that cancer cell death is a biochemically driven outcome that is subject to scientific understanding as well as rational targeting.

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CELL CARCINOMA AND OTHER TUMORS
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DECLARATION OF DR. SINAN TAS

Exhibit S

Zhang Y, *Nature* 2001;410:599-604.

Hedgehog acts as a somatic stem cell factor in the *Drosophila* ovary

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Secreted signalling molecules of the Hedgehog (Hh) family have many essential patterning roles during development of diverse organisms including *Drosophila* and humans^{1,2}. Although Hedgehog proteins most commonly affect cell fate, they can also stimulate cell proliferation. In humans several distinctive cancers, including basal-cell carcinoma, result from mutations that aberrantly activate Hh signal transduction³. In *Drosophila*, Hh directly stimulates proliferation of ovarian somatic cells^{4–6}. Here we show that Hh acts specifically on stem cells in the *Drosophila* ovary. These cells cannot proliferate as stem cells in the absence of Hh signalling, whereas excessive Hh signalling produces supernumerary stem cells. We deduce that Hh is a stem-cell factor and suggest that human cancers due to excessive Hh signalling might result from aberrant expansion of stem cell pools.

In adult *Drosophila* females, egg chambers are produced continuously in the germarium of each of the 15–18 ovarioles that are bundled together to form an ovary⁷. In regions 1 and 2a of the germarium, 16-cell germline cysts develop from germline stem cells (Fig. 1). Each cyst is enveloped by a monolayer of follicle cells in region 2b and separated from the next cyst by a short stalk as it buds from region 3 to form an egg chamber. Follicle and stalk cells derive from somatic stem cells that reside at the region 2a/2b border⁸. When a somatic stem cell divides, one daughter retains a stem cell identity and continues to divide as a stem cell for several days⁸. The other, 'pre-follicle cell' daughter proliferates for about eight cycles as its progeny associate with germline cysts, pass posteriorly down the ovariole over a 5–6-day period, and differentiate into multiple specialized cell types^{7,8}. Hedgehog (Hh) is expressed selectively in specialized non-proliferating, somatic 'terminal filament' and 'cap' cells at the anterior tip of the germarium⁴ (Fig. 1). Inactivation of Hh, using conditional *hh* alleles, arrests egg chamber budding, and causes germline cysts to accumulate in swollen germaria, suggesting that too few follicle cells are being produced⁴. Conversely, excessive Hh signalling in germarial region 2 can be induced by temporally controlled activation of an *hh* transgene or by inactivation of the Hh receptor Patched (Ptc), and causes marked overproliferation of somatic cells, which accumulate between egg chambers^{4–6} (Fig. 2).

We investigated the proliferative response to excessive Hh signal

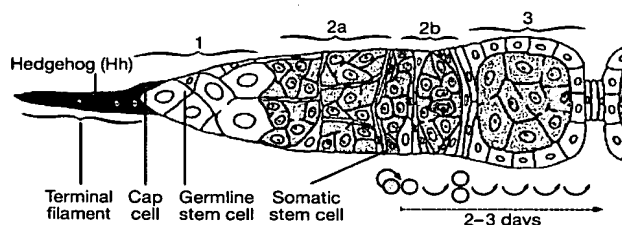


Figure 1 The *Drosophila* germarium. Regions 1 and 2a contain large proliferating germline stem cells (yellow), cystoblasts and cystocyte clusters (shaded), and smaller non-proliferating somatic cells. Somatic stem cells (green) renew and produce pre-follicle daughter cells (colourless), which divide roughly five times in the germarium (~50 h), three more times up to stage 6 (~30 h) and then arrest before differentiation, and exit from the ovariole (~30 h later). Hedgehog-producing cells and the oocyte are coloured red and blue respectively.

of 0.8 l min⁻¹. LC conditions were Vydac C₄ protein HPLC column (15 cm × 4.6 mm i.d.) using an acetonitrile:water:tetrahydrofuran gradient at 1 ml min⁻¹. For this interface, the column effluent was split using a zero-dead-volume T connector, with a split ratio of 25:1 determined by the length of a fused silica capillary on the waste side of the T. The remainder of the column effluent was fed to the transfer line of the interface. The ISP needle was maintained at 5.5 kV. High-purity nitrogen was used as the nebulizing gas at a flow rate of 0.6 l min⁻¹. A Macintosh Quadra900 computer was used for instrument control, data acquisition and data processing. A dwell time of 10 ms Da⁻¹ was used for full-scan (*m/z* 50–400) LC/MS analyses.

MS/MS measurements were based on collision-induced dissociations (CID) within the radiofrequency-only quadrupole at a collision energy of 30 eV. Argon was used as the collision gas at an indicated thickness of 220. Extraction efficiency was obtained from replicate spiking experiments and was determined ~16%.

For drought experiments, water was withheld for a total of 11 days. Leaves were harvested at the end of the 11-day drought period. Parallel control samples were harvested from well-watered plants. A 11-day drought period resulted in a significant reduction in the fresh weight/dry weight ratio compared with well-watered controls ($P < 0.001$, *t* test). Lipids were extracted from leaf samples from well-watered plants and plants subjected to a drought treatment. S1P levels were quantified by LC/MS as described above. Estimation of S1P levels in the lipid extracts was based on a linear calibration ($r^2 = 0.996$) obtained from areas under peaks of LC/MS analysis of known concentrations of authentic S1P. S1P levels are expressed in terms of dry weight of leaves based on linear calibrations of fresh weight versus dry weight from well-watered control plants ($r^2 = 1.000$) and plants subjected to the drought treatment ($r^2 = 0.999$).

Preparation of sphingolipid solutions

S1P, dihydro-S1P and DHS were obtained from Biomol (Plymouth Meeting, Pennsylvania, USA) and stock solutions were prepared in methanol (S1P and dihydro-S1P) or ethanol (DHS).

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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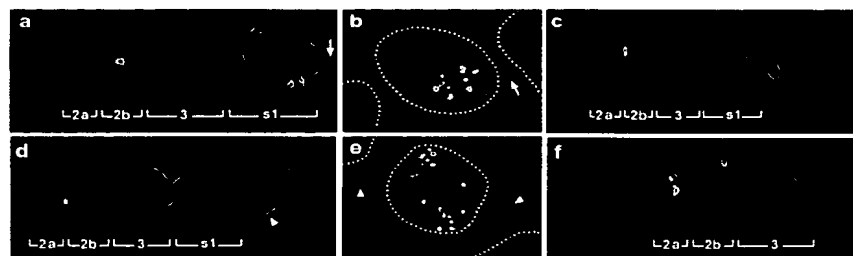


Figure 2 Phospho-histone H3 labelling of follicle cells and somatic stem cells. Wild-type ovarioles (a–c) and marked *ptc* mutant ovarioles (d–f) were labelled with antibody against phospho-histone H3 (green) and Fas III (red). The short stalks of wild-type ovarioles (arrows in a and b) are replaced by large masses of Fas III-positive cells in *ptc* mutant ovarioles (arrowheads in d and e). a, d, Mitotic cells (green) in region 2b also stain

with Fas III and are therefore not stem cells. b, e, Mitotic cells in the follicular monolayer around stage 6 egg chambers. c, f, Mitotic cells at the region 2a/2b border, anterior to the limits of Fas III staining, are putative stem cells. Ovarioles containing only *ptc* mutant cells were distinguished from mosaic ovarioles by the absence of *tub-lacZ* transgene products.

transduction further by using antibodies against phospho-histone H3, which stains cells only during mitosis⁹. We induced high levels of Hh signal transduction by inactivating *patched* (*ptc*) in marked somatic cell clones generated by heat-shock induced mitotic recombination¹⁰. Ovaries were examined 8 d later (Fig. 2) to ensure that all proliferating somatic cells assayed derived from stem cells that were present at the time of clone induction. As each ovariole contains more than one somatic stem cell⁸, this procedure generates some ovarioles containing only *ptc* mutant somatic cells and others that are mosaic for wild-type and *ptc* mutant cells. The number of mitotic somatic cells in germarial region 2 (Fig. 2a, d) in ovarioles containing only *ptc* mutant somatic cells was twice that in wild-type ovarioles (Table 1). A similar ratio was observed in region 3 of the germarium and in newly budded (stage 1–2) egg chambers (Table 1), suggesting an early increase in proliferation of *ptc* mutant cells followed by wild-type rates of proliferation of a twofold enlarged cell population. Accordingly, the follicular monolayers surrounding stage 6 egg chambers in wild-type and *ptc* mutant ovarioles (Fig. 2b, e) contained almost identical

numbers of mitotic cells within numerically equivalent cell populations (Table 1). In ovarioles mosaic for *ptc* mutant and wild-type somatic cells, the number of cells in mitosis was increased roughly 1.5-fold in region 2; again, this ratio was maintained in region 3 and in the earliest egg chambers. This is consistent with a cell-autonomous effect of *ptc* inactivation⁶, affecting roughly half of the somatic cells in a mosaic germarium.

No definitive positive marker for ovarian somatic stem cells has been described¹¹. However, when a single wild-type lineage of proliferating somatic cells is labelled by a stem cell recombination event, only one cell at the most anterior position fails to stain with Profilin¹¹ or Fasciclin III (see below), suggesting that this cell is a somatic stem cell. We therefore double-stained ovarioles with antibodies against Fasciclin III (Fas III) and phospho-histone H3, and counted the number of proliferating cells at the border of region 2a/2b that failed to stain with Fas III (Fig. 2c, f). The number of such putative stem cells in mitosis in pure *ptc* mutant ovarioles was roughly twice (0.17/0.08) that in wild-type ovarioles (Table 1). This shows that excessive Hh signal transduction promotes somatic stem cell proliferation and that this might account entirely for the ensuing somatic cell overproliferation.

To establish whether excessive Hh signalling accelerated somatic stem cell cycles or increased the number of stem cells in an ovariole, we counted stem cells by using mitotic recombination to provide up to four distinct genetic lineage labels (Fig. 3). We induced *ptc* mutant, or control, somatic stem cell clones in a genotype that allowed a loss of *tub-lacZ* and the gene for green fluorescent protein (*GFP*) transgenic markers by independent heat-shock-induced recombination events. We then waited 3 d for *ptc* inactivation to

Table 1 Number of mitotic somatic cells per ovariole

Ovarioles	Stem cell	Region 2b	Region 3	Stage 1–2	Stage 6*
Wild-type	0.08	0.6	0.8	1.4	11.7
All <i>ptc</i> [−]	0.17†	1.2‡	1.5‡	2.8‡	10.9
Mosaic <i>ptc</i> [−]	0.14	1.0	1.2	2.3	

Each number is the mean from 97 or more ovarioles.

*Only the monolayer of follicle cells surrounding stage 6 egg chambers was examined.

† $P = 0.052$ in comparison with wild-type.

‡Significantly different from wild-type value ($P < 0.01$).

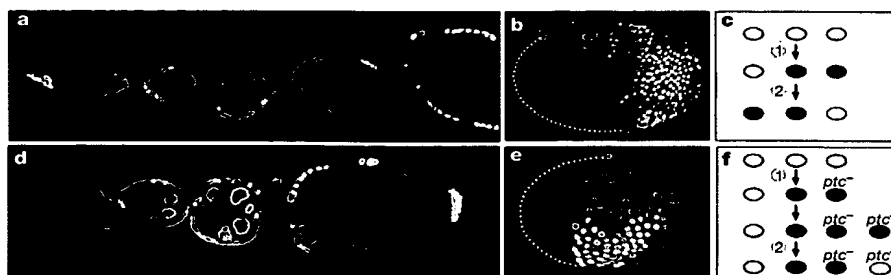


Figure 3 Counting somatic stem cells in wild-type and *ptc* mutant ovarioles. Stem cell clones generated in wild-type (a–c) and *ptc* heterozygous flies (d–f) stained for expression of *lacZ* (red) and *GFP* (green). Wild-type ovarioles (a) and some individual wild-type egg chambers (b) contained up to three distinguishable follicle cell types, whereas some *ptc* mutant ovarioles (d) or even single egg chambers (e) included four distinguishable follicle cell types. c, f, Examples of stem cell recombination events

induced by the indicated heat shocks (1 and 2) that generate three-colour wild-type ovarioles (c), and four-colour *ptc* mutant ovarioles (f) from three *lacZ*⁺ *GFP*⁺ (red plus green staining) stem cells. Germline cell nuclei are visible in some optical sections shown (green anterior cells in a, several in d) but are readily distinguished from follicle cells by their larger size.

Table 2 Distribution of distinguishable somatic stem cell genotypes in wild-type and *ptc* mutant ovarioles

Ovarioles	Distinguishable genotypes	8 d†	10 d	12 d	14 d	16 d*
Wild-type	1	16	21	37	41	48
	2	57	53	57	52	51
	3	27	26	6	7	1
<i>ptc</i>	1	14	21	24	35	65
	2	30	47	64	61	29
	3	42	29	9	4	6
	4	14	3	3	0	0

Each entry is expressed as the percentage of ovarioles with the stated number of distinguishable genotypes. At least 100 ovarioles were counted for each entry.

*Distribution of ovarioles significantly different between wild-type and *ptc* by χ^2 test ($P < 0.05$).

†Random recombination events (with overall efficiency of 73% over two heat shocks) predict a distribution of 20% (one genotype), 60% (two genotypes), 25% (three genotypes) for three stem cells.

affect stem cell behaviour and heat-shocked animals again to ensure that all stem cells, including any newly engendered by loss of *ptc*, could undergo recombination events distinguishing them from other stem cells in the same ovariole. After 8 d, the number of distinguishable somatic cell genotypes (*lacZ*⁺ *GFP*⁺, *lacZ*⁺ *GFP*⁺, *lacZ*⁺ *GFP*⁺ and *lacZ*⁺ *GFP*⁺) in a single ovariole was scored (Fig. 3). This provides a minimum measure of the number of somatic stem cells present at the time of the second heat shock. Wild-type ovarioles contained up to three distinguishable genotypes, implying the presence of up to three somatic stem cells in a single ovariole (Fig. 3a–c). Indeed, the observed distribution of marked genotypes indicates that most or all wild-type ovarioles contain three somatic stem cells (Table 2). Four distinguishable somatic cell genotypes were never observed in wild-type ovarioles but were present in 14% of *ptc* mutant ovarioles (Fig. 3d–f; Table 2), showing that the latter

contained at least four somatic stem cells. The constraints of the experiment require that these 'four stem cell ovarioles' contain two wild-type stem cells and two *ptc* mutant stem cells (Fig. 3f). We therefore deduce that such ovarioles originally included a single *ptc* mutant stem cell that duplicated within 3 d to form two stem cells. We are confident that the different follicle cell genotypes observed do indeed derive from recombination events in stem cells (and therefore reflect stem cell number) because four genotypes were evident in some *ptc* mutant ovarioles as long as 12 d after heat-shock (Table 2) and spontaneous recombination events in these flies were infrequent.

It has previously been estimated that there are two, rather than three, somatic stem cells in a wild-type ovariole, by counting the progeny of single marked stem cells as constituting roughly 50% of the follicle cells in an ovariole⁸. There are several possible explanations for this discrepancy. Later, we present further evidence that in our studies wild-type ovarioles contained three stem cells.

To reveal the effects of *ptc* inactivation on stem cell number directly we positively marked single wild-type and *ptc* mutant stem cell lineages with *lacZ* activity^{8,12} (Fig. 4b), and we counter-stained with antibody against Fas III (Fig. 4). We used a very mild heat-shock treatment (37 °C for 10 min) to induce mitotic recombination so that marked ovarioles generally contained only a single *lacZ*⁺ clone, and we examined ovaries 5 d later to ensure that clones were of stem cell origin. In wild-type ovarioles, most *lacZ*⁺ clones (32/46) included only a single cell at the region 2a/2b border that did not stain with Fas III (Fig. 4c); only a single clone included two such cells (Table 3). This is consistent with the idea that *lacZ*⁺ *Fas III*[−] somatic cells at the region 2a/2b border are indeed stem cells. In *ptc* heterozygous animals, some recombinant *lacZ*⁺ stem cell clones also lost *ptc* activity through a second recombination event (Fig. 4b). These *ptc* mutant stem cell clones were identified by their over-proliferation and accumulation between egg chambers. Most of the *ptc* mutant clones (31/41) included two *lacZ*⁺ *Fas III*[−] cells at the region 2a/2b border (Fig. 4d, f); only one *ptc* mutant clone included a single such putative stem cell (Table 3). The two *ptc* mutant stem cells must, in most cases, have derived from a single recombination event that initially generated a single *ptc* mutant stem cell. Thus, in almost every ovariole examined, a loss of *ptc* activity led to a

Table 3 Numbers of wild-type and *ptc* mutant lineages with zero, one and two stem cells

Ovarioles	Two stem cells	One stem cell	No stem cells
Wild-type	1	32	13
<i>ptc</i>	31	1	9

A small number of ovarioles could not be scored definitively because some somatic cells in region 1 and early region 2 (generally described as non-proliferating) expressed β -galactosidase.

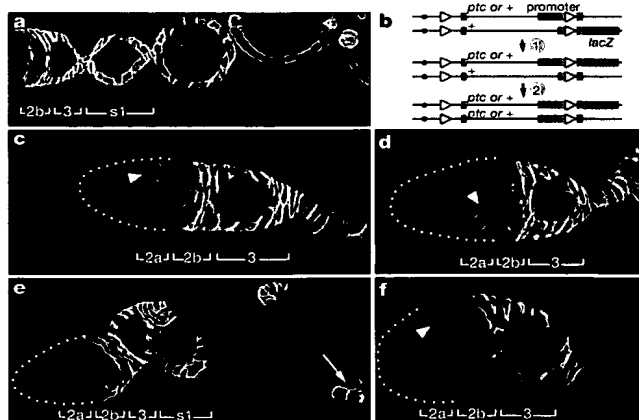


Figure 4 Direct labelling of wild-type and *ptc* mutant somatic stem cells. Ovarioles were stained for *lacZ* expression (red) and Fas III (green) 5 d after induction of mitotic recombination in *ptc* heterozygotes or control flies by a single heat shock. **a**, In control ovarioles *lacZ*⁺ clones are induced by event 1 depicted in **b** and populated both a fraction of the germarium and the epithelium of several egg chambers from stage 1–6. **c**, Wild-type stem cell clones generally included a single *lacZ*⁺ *Fas III*[−] cell at the region 2a/2b border. **e**, Occasionally no such putative stem cell was present. **b**, In *ptc* heterozygotes the generation of marked *ptc* mutant clones requires two mitotic recombination events (1 and 2) in the same cell. **d**, **f**, Such clones, identified by *lacZ*⁺ cells in enlarged stalks (not shown), almost invariably included two *lacZ*⁺ *Fas III*[−] putative stem cells, either adjacent (**d**) or not (**f**; greater separations were also seen).

Table 4 Percentage of *lacZ*⁺, mosaic and *lacZ*[−] ovarioles in wild-type, *smo* and *ptc* heterozygotes

Ovarioles	Genotype	Days after heat shock				
		3	8	10	12	14
Wild-type	<i>lacZ</i> ⁺	0	44	51	61	60
	Mosaic	100	56	49	36	32
	<i>lacZ</i> [−]	0	0	2	3	8
<i>smo</i>	<i>lacZ</i> ⁺	0	87	94	100	100
	Mosaic	100	13	6	0	0
	<i>lacZ</i> [−] (<i>smo</i> [−])	0	0	0	0	0
<i>ptc</i>	<i>lacZ</i> ⁺	0	32	33	33	34
	Mosaic	100	57	51	39	31
	<i>LacZ</i> [−] (<i>ptc</i> [−])	0	11	15	29	35

Each entry derives from at least 100 ovarioles of adults after heat-shock-induced mitotic recombination in *lacZ*⁺/*lacZ*[−] third-instar larvae.

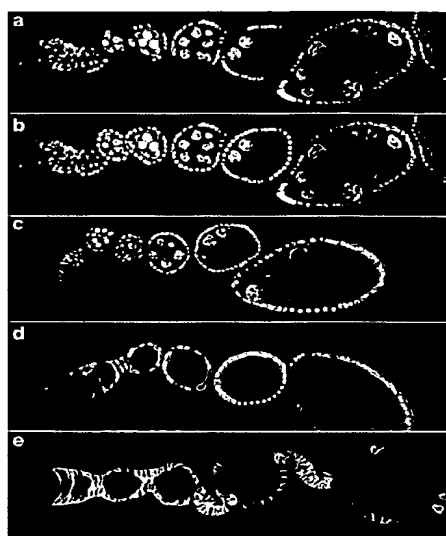


Figure 5 Somatic stem cell proliferation requires *smo* and *ci* activities. **a–d**, Wild-type clones (**a**, **b**) *smo* mutant clones (**c**) and *ci* mutant clones (**d**) were marked by loss of a *tub-lacZ* transgene and were identified 8 d later by the absence of *lacZ* expression (colourless nuclei, artificially coloured green for clarity in **b–d**). Panels **a** and **b** show the same ovariole. **e**, Fas III staining highlights excessive cells between egg chambers resulting from the expression of an activated *ci* transgene initiated 8 d earlier.

cell-autonomous doubling of somatic stem cell number. In 19 of these instances, the two *ptc* mutant stem cells were directly adjacent (Fig. 4d), suggesting that excessive Hh signal transduction allowed local expansion of a stem cell niche. In the remaining 12 cases the additional *ptc* mutant stem cell had migrated away from its presumed sister cell (Fig. 4f). In several wild-type and *ptc* mutant ovarioles, no stem cell was visible for a *lacZ*⁺ lineage (Fig. 4e; Table 3). We presume that in these cases the marked stem cell died or started to proliferate like a pre-follicle cell within the 5-d period between clone induction and ovary dissection.

We investigated whether Hh is required for somatic stem cell maintenance or proliferation by using conditional *hh* alleles and by generating somatic cell clones lacking *smoothed* (*smo*) activity. Inactivation of *smo* universally blocks Hh signal transduction cell-autonomously¹. Control and *smo* mutant clones, each marked by loss of a *tub-lacZ* transgene, were examined at various times after induction. At 3 d, *smo* mutant clones of the same size as control clones were efficiently recovered in egg chambers of all stages (Table 4). However, by 8 d, when only stem cell clones should remain in the ovariole, very few *smo* mutant clones were observed. The *smo* mutant clones that were present generally occupied only a portion of a single egg chamber (Fig. 5c), in contrast to a typical control stem cell clone, which spread throughout the ovariole (Fig. 5a, b). By 12 d after clone induction no *smo* mutant clones were observed (Table 4). These results demonstrate that a cell that is unable to transduce a Hh signal cannot proliferate as a somatic stem

Table 6 Percentage of mosaic ovarioles induced in wild-type adults of different ages

Age (d)	0	2	4	6	8	10	12
Mosaic (%)	83	83	84	85	87	85	81

Clones were induced in *lacZ*⁺/*lacZ*⁻ adults at the given times after eclosion and the percentage of mosaic ovarioles was scored 8 d later in a sample of at least 100 ovarioles.

(cell). We cannot be certain of the fate of such cells, but we suspect that *smo* mutant somatic stem cells remain abnormally quiescent for up to 7–8 d and at some point during this period acquire the characteristics of a pre-follicle cell, proliferating normally in that capacity to produce a clone occupying roughly one-third of an egg chamber.

In *hh*^{ts2} animals shifted to the restrictive temperature of 29°C a statistically significant reduction in the number of mitotic cells labelled by phospho-histone H3 antibody was seen in region 2b by 72 h, and in germarial region 3 by 96 h (Table 5). Double-staining with Fas III antibody showed that the number of stem cells undergoing mitosis was already reduced by 48 h and was undetectable by 72 h. These reductions were not statistically significant because wild-type ovarioles sampled at these time points collectively contained only two or three somatic stem cells. Nevertheless, these mitotic labelling data are the first direct evidence that Hh inactivation reduces somatic cell proliferation and are consistent with a primary effect on stem cell proliferation.

The effects of Hh signalling on cell fate determination in *Drosophila* are mediated largely by altering the activity of the transcription factor Cubitus interruptus (Ci)¹. We examined the role of Ci in somatic stem cell proliferation first by inducing somatic clones lacking *ci* activity. As with *smo*, very few clones were recovered 8–10 d after clone induction and these clones occupied only a small proportion of the ovariole (Fig. 5d), indicating that stem cells cannot proliferate normally in the absence of *ci* activity. When we induced the expression of a constitutively active derivative of Ci (ref. 13) by heat-shock-induced excision of a transcriptional terminator, we recovered ovarioles showing massive overproliferation of somatic cells, which accumulated between egg chambers (Fig. 5e) as observed for *ptc* mutant ovarioles. Thus, the activity of Hh as a stem cell factor seems to depend on Ci-mediated regulation of transcription. The definition of relevant target genes presents an avenue into understanding how stem cell identity and proliferation are regulated.

Somatic stem cells seem to have a limited lifespan⁸. We investigated the longevity of wild-type ovarian somatic stem cells by inducing marked clones in third-instar larvae and examining adult ovaries 8–14 d later. The proportion of mosaic ovarioles declined from 56% (in 3-day-old adults) to 32% (in 9-day-old adults) during this period (Table 4), indicating a loss of some of the stem cells present at the time of clone induction. To see whether these stem cells were being replaced we performed a complementary experiment in which clones were induced in adults of various ages and then assayed 8 d later. The frequency of mosaic ovarioles was unchanged in adults from zero to 12 d after eclosion (Table 6), suggesting that the total number of somatic stem cells was constant over this period. We therefore deduce that, on average, wild-type stem cells are lost roughly every 8–9 d but are subsequently

Table 5 Number of mitotic somatic cells per ovariole*

Days at 29°C	+/+					<i>hh</i> ^{ts2} / <i>hh</i> ^{ts2}					<i>hh</i> ^{ts2} /+				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Stem cell	0.02	0.06	0.04	0.06	0.06	0.02	0.08	0.02	0.00	0.00	0.02	0.06	0.04	0.02	0.02
Region 2b	0.46	0.60	0.60	0.54	0.44	0.36	0.66	0.36	0.16*	0.22	0.62	0.74	0.46	0.36	0.30
Region 3	0.66	1.28	1.36	1.08	0.98	0.70	0.94	0.94	0.77	0.48*	0.82	1.12	0.98	0.72	0.58
Stage 1–2	2.51	3.12	4.22	3.76	3.90	2.68	2.42*	2.72*	2.14*	1.30*	2.44	2.58	2.98	2.76	1.92

Each number is the mean from 50 ovarioles, scored every 24 h after transfer of 2-day-old adults from permissive (18°C) to restrictive (29°C) temperature.

*Significantly different from +/+ values (*P* < 0.05).

replaced by division of another stem cell in the same ovariole to produce two sister stem cells.

We also note that the distribution of genotypes in wild-type ovarioles at day 8 (Table 4), before significant stem cell loss and replacement, is consistent with the presence of three somatic stem cells. A simple model of random, independent conversion of each of three stem cells to a *lacZ*⁺ genotype with probability $P = 0.5$ predicts a distribution (*lacZ*⁺ 42%; mosaic 56%; *lacZ*⁻ 2%) similar to that observed. This model would predict a much higher proportion of *lacZ*⁺ ovarioles relative to mosaic ovarioles for all P values if only two somatic stem cells were present.

When marked *ptc* mutant *lacZ*⁻ stem cell clones were induced in third-instar larvae, the number of ovarioles containing only *lacZ*⁺ cells remained roughly constant from 8 to 14 d later (Table 4), indicating that *ptc* mutant stem cells were not readily lost from mosaic ovarioles. Conversely, there was a strong preference for mosaic ovarioles to become populated entirely by *ptc* mutant cells over time. For example, 14 d after clone induction, 35% of ovarioles were composed entirely of *ptc* mutant *lacZ*⁻ clones, whereas in wild-type animals only 8% of ovarioles contained solely *lacZ*⁺ somatic cells (Table 4). The stability and apparently aggressive behaviour of *ptc* mutant clones is likely to be due in some part to the ability of *ptc* mutant stem cells to duplicate soon after they arrive in a vacated stem cell niche. However, it is possible that *ptc* mutant stem cells also actively displace wild-type stem cells from their niches.

All of our observations can most easily be rationalized by postulating that there are only a limited number of positions (literally three) within a germarium where somatic cells receive the correct combination of extracellular signals to instruct them to behave as stem cells. In a wild-type ovariole one of the two daughters of a stem cell division necessarily leaves this environment and therefore does not adopt a stem cell fate. Occasionally, a stem cell might leave its niche and proliferate as a pre-follicle cell. The vacant niche becomes occupied shortly afterwards by the daughter of another stem cell. In this model the division of stem cells is not intrinsically asymmetric. Rather, the different behaviour of the two daughters is dictated by their physical environment, so that both daughters can become stem cells if two stem cell niches are vacant. Hh seems to be one of the key environmental signals for directing somatic stem cell behaviour. Proliferation of a stem cell requires Hh signal transduction; excessive Hh signal transduction allows expansion of the stem cell niche. Our results suggest that each stem cell niche can accommodate two stem cells if these cells are undergoing excessive Hh signal transduction.

Because Hh signalling, like all major signalling pathways, is used in a large number of contexts, there is no compelling reason to expect that the proliferative effects of Hh proteins are always targeted to stem cells. However, several characteristics of normal and deregulated development of vertebrate epidermis and hair follicles prompt us to suggest that Sonic hedgehog (Shh) might also act on stem cells in this context. Shh is required for hair follicle development in mice, and *Shh* mutant phenotypes include reduced proliferation of ectodermal stem cell derivatives^{14,15}. Conversely, inappropriate mutational activation of the Shh signal transduction pathway is universally observed in human familial and sporadic basal-cell carcinomas (BCCs)^{16–20}. Several observations suggest that activation of this pathway might be obligatory in the aetiology of BCC and can suffice to initiate this cancer^{16,21–24}. The cellular origin of BCC has not been defined, but outer root sheath cells of hair follicles, which include epidermal and follicular stem cells in a specialized bulged region^{25–27}, are strongly implicated²⁸. In the light of our present studies, we suggest that proliferation of follicular stem cells might normally require Shh, among other factors, and that mutational activation of the Shh signalling pathway in BCC acts specifically on these stem cells to increase their cycling frequency or their cell number, or perhaps even to expand their niche into or towards the surrounding epithelium. Any of these changes could

produce a sustainable large increase in a population of transiently proliferating cells, which would constitute the bulk of the carcinoma. At present these hypotheses concerning Hh-associated cancers are speculative and remain to be tested. □

Methods

Mutant *ptc* and *smo* clones marked by the absence of a *tub-lacZ* transgene were generated as described previously⁹ by using a standard heat shock of 60 min at 39 °C. To count stem cells, clones were induced in flies of genotype *hsp70-flp/+; FRT42D ptc²² [or +]/FRT42D P[tub-lacZ]; FRT82B P[hsp70-myc-GFP]/FRT82B*. To mark stem cell lineages with *lacZ*⁺ we used flies of genotype *hsp70-flp/+; FRT42D ptc²² [or +] X15-29/FRT42D X15-31* (ref. 12). Clones lacking *ci* activity and marked by the loss of GFP were generated by using flies of genotype *hsp70-flp/hsp70-flp; FRT42D P[ci⁺] P[hsp70-GFP]/FRT42D; ci²⁴/ci²⁴* (ref. 29). Clones expressing activated *ci* were generated by excision of an FRT-flanked *CD2* gene from an *act5C > CD2 > GAL4* transgene²⁹ in the presence of the activated *UAS-Ci5m* transgene from line T5m-s1 (ref. 13). Ovaries were stained as described previously for β -galactosidase, Fas III and phospho-histone H3 epitope⁶, except that secondary antibodies for double-staining used Alexa fluorescence probes (Molecular Probes). GFP encoded by an *hsp70-myc-GFP* transgene³⁰ was detected by direct fluorescent imaging after a heat shock (60 min, 39 °C) and a 3-h recovery period.

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Structural basis for co-stimulation by the human CTLA-4/B7-2 complex

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Regulation of T-cell activity is dependent on antigen-independent co-stimulatory signals provided by the disulphide-linked homodimeric T-cell surface receptors, CD28 and CTLA-4 (ref. 1). Engagement of CD28 with B7-1 and B7-2 ligands on antigen-presenting cells (APCs) provides a stimulatory signal for T-cell activation, whereas subsequent engagement of CTLA-4 with these same ligands results in attenuation of the response¹. Given their central function in immune modulation, CTLA-4- and CD28-associated signalling pathways are primary therapeutic targets for preventing autoimmune disease, graft versus host disease, graft rejection and promoting tumour immunity^{1,2}. However, little is known about the cell-surface organization of these receptor/ligand complexes and the structural basis for signal transduction. Here we report the 3.2-Å resolution structure of the complex between the disulphide-linked homodimer of human CTLA-4 and the receptor-binding domain of human B7-2. The unusual dimerization properties of both CTLA-4 and B7-2 place their respective ligand-binding sites distal to the dimer interface in each molecule and promote the formation of an alternating arrangement of bivalent CTLA-4 and B7-2 dimers that extends throughout the crystal. Direct observation of this CTLA-4/B7-2 network provides a model for the periodic organization of these molecules within the immunological synapse and suggests a distinct mechanism for signalling by dimeric cell-surface receptors.

The specificity of the T-cell response depends on the engagement of the T-cell receptor (TCR) with the cognate major histocompatibility complex (MHC)-peptide complex on the surface of APCs³. Effective regulation of this response requires a number of antigen-independent co-stimulatory signals, the most important of which are provided by the related T-cell surface molecules, CTLA-4 and CD28 (approximately 30% identity)¹. Studies indicate that CTLA-4 both disrupts stimulatory signalling complexes by competing with CD28 for binding the B7 isoforms, and promotes the assembly of inhibitory signalling complexes⁴. To examine the structural basis for co-stimulation, we have solved the structure of the complex formed by the disulphide-linked homodimer of human CTLA-4 and the receptor-binding domain of human B7-2. The asymmetric unit contains two monomers of each CTLA-4 and B7-2, providing two independent views of the CTLA-4/B7-2 interface. Notably, crystal packing generates an alternating network of bivalent CTLA-4 and B7-2 homodimers. The CTLA-4 and B7-2 monomers are both two-layer β -sandwiches that display the chain topology characteristic of the immunoglobulin variable (V-type) domains present in antigen

receptors such as the TCR β -chains and antibody V_L and V_H domains⁵. The front and back sheets of CTLA-4 contain strands A'GFCC' and ABEDC', respectively, whereas the front and back sheets of B7-2 are composed of AGFCC'C' and BED strands, respectively. The individual CTLA-4 monomers in the present crystal structure are highly similar to the monomers observed in the murine CTLA-4 crystal structure⁶ and the human CTLA-4 NMR structure⁷, with root mean square (r.m.s.) deviations of 1.6 Å and 2.2 Å, respectively. Furthermore, the structure of the B7-2 monomer is similar to that observed for both unliganded B7-1 (ref. 8) and B7-2 (X.Z. and J.-C.D.S., unpublished results), displaying r.m.s. deviations of 2.3 Å and 0.8 Å, respectively.

The CTLA-4/B7-2 binding interface is formed by packing interactions between the front sheet of each molecule (Fig. 1a). CTLA-4 makes extensive contribution of residues from the FG loop (the complementary determining region 3 (CDR3)-like segment) and to a lesser extent the C and C' strands. These segments pack against the G, F, C, C' and C'' strands of B7-2, which form a shallow concave surface as a consequence of the natural twist of the sheet. The two independent CTLA-4/B7-2 interfaces have very similar atomic contacts, and bury 1,290-Å² (interface A) and 1,212 Å² (interface B) of total accessible surface area. The size of this interface compares favourably with other complexes of V-type immunoglobulin domains, such as the heterophilic CD2/CD58 complex⁹ and homophilic E-cadherin adhesion dimer¹⁰ that bury 1,160 Å² and 1,300 Å², respectively. Although the CD2/CD58 and E-cadherin interfaces are formed by packing of the strands residing on the front sheet of each interacting molecule, the CTLA-4/B7-2 interface is formed by the β -strands of B7-2 and surface loops on CTLA-4. In the crystal, the two CTLA-4/B7-2 interfaces show a difference in geometric complementarity, with shape correlation statistics (Sc values) of 0.66 and 0.58 for the A and B interfaces, respectively (see Methods). The differences in buried surface area and surface complementarity observed in the two CTLA-4/B7-2 interfaces arise from an approximate 13° rotational difference between the two CTLA-4 molecules relative to their respective B7-2 partners (see Methods).

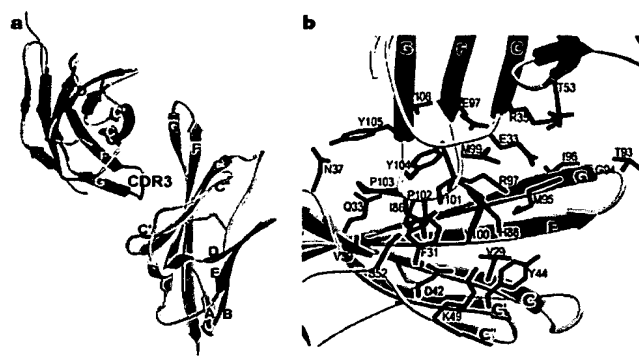


Figure 1 The CTLA-4/B7-2-binding interface. **a**, Ribbon diagram of the CTLA-4/B7-2 monomers that form the binding interface. For CTLA-4, the strands are labelled A (residues 4–8), A' (residues 11–13), B (residues 19–26), C (residues 33–42), C' (residues 45–54), C'' (residues 56–60), D (residues 67–72), E (residues 75–81), F (residues 90–99) and G (residues 105–109; 112–115). The A'GFCC' (front) and ABEDC' (back) sheets are coloured pink and red, respectively. The CDR3-like loop of CTLA-4 is labelled. For B7-2, the strands are labelled A (residues 3–7), B (residues 9–11), C (residues 27–35), C' (residues 38–45), C'' (residues 47–49), D (residues 60–64), E (residues 67–73), F (residues 81–91) and G (residues 94–108). The AGFCC'C' (front) and BED (back) sheets are coloured dark blue and blue, respectively. All inter-sheet disulphides are shown in yellow. **b**, Detailed view of the human CTLA-4/B7-2 interface. The interface is formed by residues from the front sheets of CTLA-4 (CDR3 and the C and C' strands) and a concave surface on B7-2 (the G, F, C, C' and C'' strands and the CC', C'D and FG loops).